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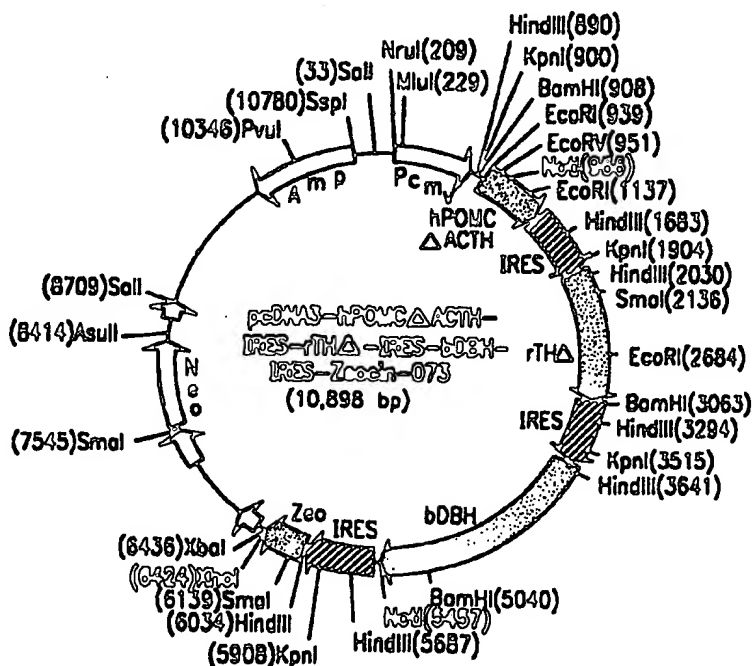
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(54) Title: CELL LINE PRODUCING ANALGESIC COMPOUNDS FOR TREATING PAIN**(57) Abstract**

A genetically engineered cell line that produces at least one catecholamine, at least one endorphin, and at least one enkephalin, for the treatment of pain. The cells may be provided directly to a patient in need thereof, or encapsulated to form a bioartificial organ.



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Cell line producing analgesic compounds for treating pain

Field of the Invention

The present invention relates to a cell line
5 useful for the treatment of pain. More particularly,
the cell line of this invention has been genetically
engineered to produce at least one analgesic compound
from each of the groups consisting of endorphins,
enkephalins, and catecholamines.

10 Background of the Invention

Pain is a common symptom of disease. The
superficial dorsal horn of the spinal cord, where
primary afferent fibers carrying nociceptive
information terminate, contains enkephalinergic
15 interneurons and high densities of opiate receptors.
In addition, there is a dense concentration of
noradrenergic fibers in the superficial laminae of the
spinal cord.

Acute pain arises in response to acute
20 noxious stimuli. Chronic pain is predominantly due to
neuropathies of central or peripheral origin. This

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neuropathic pain is the result of aberrant somatosensory processing that can result in increased sensitivity to a painful stimulus (hyperalgesia) and pain associated with a stimulus that does not usually
5 provoke pain (allodynia).

Intrathecal injection of morphine into the spinal subarachnoid space produces potent analgesia. Similarly, intrathecal administration of norepinephrine or noradrenergic agonists also produces analgesia.
10 See, e.g., Sagen et al., Proc. Natl. Acad. Sci. USA, 83, pp. 7522-26 (1986).

Co-administration of subeffective doses of opiates, such as enkephalins, and catecholamines, such as norepinephrine, may synergize to produce analgesia.
15 Ibid. Chromaffin cells in the adrenal medulla produce and release several neuroactive substances including norepinephrine, epinephrine, met-enkephalin, leu-enkephalin, neuropeptide Y, vasoactive intestinal polypeptide, somatostatin, neurotensin, cholecystokinin
20 and calcitonin gene-related peptide. See, e.g., Sagen et al., Proc. Natl. Acad. Sci. USA, 83, pp. 7522-26 (1986); Sagen et al., Jour. Neurochem., 56, pp. 623-27 (1991).

Because chromaffin cells produce both opioid
25 peptides and catecholamines, one approach to reduction of nociceptive response or pain sensitivity has investigated transplanting adrenal medullary tissue, as well as isolated adrenal chromaffin cells, directly into CNS pain modulatory regions, in attempts to
30 provide analgesia. See, e.g., Sagen et al., Brain Research, 384, pp. 189-94 (1986); Vaguero et al., Neuroreport, 2, pp. 149-51 (1991); Ginzberg and

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Seltzer, Brain Research, 523, pp. 147-50 (1990); Sagen et al., Pain, 42, pp. 69-79 (1990).

Attempts to produce analgesic have been made using both allogeneic and xenogeneic chromaffin tissue or cells transplants. Allograft tissue is in limited supply, and is not readily available, particularly for in human pain treatment programs. In addition, allogeneic human tissue carries the risk of pathogenic contamination. See e.g., Hama and Sagen, Brain Research, 651, pp. 183-93 (1994).

Xenogeneic donors may provide large quantities of material that can be readily obtained. For this reason, bovine adrenal tissue has been used. See, e.g., Hama and Sagen, Brain Research, 651, pp. 183-93 (1994).

However, potentially serious host consequences, as well as ultimate graft rejection, are inherent problems in transplantation between disparate species. Complete graft rejection of whole or dissociated tissue may occur even in the CNS, normally thought to be immunologically privileged, due to presence of highly antigenic cells in the xenografts, particularly endothelial cells. In addition, the donor tissue must be carefully screened to avoid introduction of viral contaminants, or other pathogens, to the host. To overcome graft rejection, immunosuppression is required typically using cyclosporine A.

Some reduction in pain sensitivity has been reported resulting from these transplants, particularly for the reduction of low intensity chronic pain. In most reports, significant differences between control and transplanted animals were noted only after nicotine

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administration to stimulate opioid peptide production. However, there have been some reports that analgesia has been observed in a rat chronic pain model from basal level activity of chromaffin tissue allografts.

- 5 See, e.g., Vaquero et al., NeuroReport, 2, pp. 149-51 (1991) and Hama and Sagen, Brain Research, 651, pp. 183-93 (1994).

Bovine adrenal chromaffin cells have been encapsulated to form a bioartificial organ ("BAO") for
10 implantation into rats for the treatment of acute and chronic pain. See, e.g., Sagen et al., J. Neurosci., 13, pp. 2415-23 (1993) and Hama et al., 7th World Congress Pain, Abstract 982, Paris France (1993). Initial trials in human subject have been conducted
15 using encapsulated bovine chromaffin cells. See, Aebischer et al., Transplantation, 58, pp. 1275-77 (1994).

There have also been attempts to induce antinociception using other cells, e.g., AtT-20 cells.
20 AtT-20 cells were originally derived from a mouse anterior pituitary tumor. These cells synthesize and secrete β -endorphin. See, e.g., Wu et al., J. Neural Transpl. & Plasticity, 5, pp. 15-26 (1993). AtT-20/hENK cells are AtT-20 cells that have been
25 genetically engineered to carry the entire human pro-enkephalin A gene (i.e. containing 6 met-enkephalin sequences and one leu-enkephalin sequence) with 200 bases of 5'-flanking sequence and 2.66 kilobases of 3'-flanking sequence. See Wu et al., supra, Comb et al.,
30 EMBO J., 4, pp. 3115-22 (1985).

Wu et al., J. Neural Transpl. & Plasticity, 5, pp. 15-26 (1993) refers to rat hosts transplanted

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with AtT-20 or AtT-20/hENK cells. Unstimulated AtT-20/hENK cells produced more antinociception (tail flick test) than produced by AtT-20 implants. In contrast, isoproterenol stimulation produced more antinociception
5 with AtT-20 cells than with AtT-20/hENK cells. Ibid.

In mice hosts, AtT-20 or AtT-20/hENK implants did not affect basal response to thermal nociceptive stimuli. Mice receiving AtT-20 implants developed tolerance to β -endorphin and a μ -opioid agonist
10 (DAMGO). Mice receiving AtT-20/hENK implants developed tolerance to an δ -opioid agonist (DPDPE). In response to repeated doses of an μ opiate agonist, mice receiving AtT-20/hENK implants developed less tolerance compared to mice receiving AtT-20 cells or controls.

15 The antinociceptive effect of isoproterenol treatment appeared equal in mice receiving AtT-20 or AtT-20/hENK cell implants. See, Wu et al., J. Neuroscience, 14, pp. 4806-14 (1994). Wu et al. speculated that one reason for the absence of
20 additional antinociception in mice implanted with enkephalin producing AtT-20/hENK cells may be due to lack of sensitivity of the behavioral assays. Another possible reason was that met-enkephalin's known antagonist effect on morphine induced antinociception
25 offset the potentiating effect of the single leu-enkephalin, particularly since there are 6 met-enkephalin sequences for each leu-enkephalin sequence in pro-enkephalin A.

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Summary of the Invention

The present invention provides a cell line that has been genetically engineered to produce at least one analgesic compound from each of the groups
5 consisting of endorphins, enkephalins, and catecholamines. The cell line may be used in the treatment of pain.

There are advantages to using a cell line over the use of primary cells. Expensive and time
10 consuming testing to ensure safety and performance criteria for cells must be performed for individual isolations of primary cells. Less testing is required of a cell bank. There is no need to isolate primary cells. Output of the desired analgesics may be more
15 stable since the performance of primary cells may be dependent on the age, sex, health or hormonal status of the donor animal. It is also possible to achieve higher output of the desired products, as well as to engineer specifically modified peptides into the cell
20 line. This permits delivery of multiple analgesics simultaneously. Expression of one or more of the analgesics can be regulated (by using a regulatable promoter to drive expression). In addition, for safety, a "suicide" gene can be incorporated into the
25 cell line. Further, for encapsulation purposes proliferating cells have the advantage that they divide to replace dying or dead cells.

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Brief Description of the Drawing

Figure 1 is a plasmid map of vector pBS-hPOMC-027, pBS-IgSP-hPOMC-028 and pBS-IgSP-hPOMC- Δ ACTH-029.

5 Figure 2 is a plasmid map of vectors pCEP4-hPOMC-030, pCEP4-hPOMC-031, pcDNA3-hPOMC-034 and pcDNA3-hPOMC-035.

Figure 3 is a plasmid map of vectors pCEP4-hPOMC- Δ ACTH-032, pCEP4-hPOMC- Δ ACTH-033, pcDNA3-hPOMC-10 Δ ACTH-36 and pcDNA3-hPOMC- Δ ACTH-037.

Figure 4 is a plasmid map of vectors pcDNA3-rTH-044, pcDNA3-rTH Δ -045, and pcDNA3-rTHDKS-075 (also represented as pcDNA3-rTH Δ KS-075).

Figure 5 is a plasmid map of vectors pcDNA3-15 rTH Δ -IRES-bDBH-088 and pcDNA3-rTH Δ KS-IRES-bDBH-076.

Figure 6 is a plasmid map of vector pZeo-Pcmv-rTH Δ KS-IRES-bDBH-088.

Figure 7 is a plasmid map of vector pBS-Pcmv-rTH Δ IRES-bDBH-067.

20 Figure 8 is a plasmid map of vector pBS-hPOMC- Δ ACTH-IRES-rTH Δ IRES-bDBH-068.

Figure 9 is a plasmid map of vector pcDNA3-hPOMC- Δ ACTH-IRES-rTH Δ -IRES-bDBH-069.

Figure 10 is a plasmid map of vector pcDNA3-25 IRES-Zeocin-072.

Figure 11 is a plasmid map of vector pcDNA3-hPOMC- Δ ACTH-IRES-rTH Δ -IRES-bDBH-IRES-Zeocin-073.

Figure 12 is a plasmid map of vector pcDNA3-hPROA+KS-091.

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Detailed Description of the Invention

In order that this invention may be more fully understood, the following detailed description is set forth.

5 Any suitable cell may be transformed with the recombinant DNA molecules of this invention. Among the contemplated cells are chromaffin cells, including conditionally immortalized chromaffin cells such as those described in WO 96/02646, Neuro-2A, PC12, PC12a,
10 SK-N-MC, AtT-20, and RIN cells including RINa and RINb. Preferably the cell has endogenous prohormone convertases and/or dopa decarboxylases.

SK-N-MC cells, a neuroepithelioma cell line, co-expresses several neuropeptides, including
15 enkephalin, cholecystokinin and gastrin-releasing peptide. See, e.g., Verbeeck et al., J. Biol. Chem., 265, pp. 18087-090 (1990). The pro-enkephalin A gene has been expressed in SK-N-MC cells. See, e.g., Folkesson et al., Mol. Brain Res., 3, pp. 147-54
20 (1988). We prefer AtT-20 and RIN cells, most preferably RIN cells.

RIN cells are a pancreatic endocrine cell line derived from rat. See, e.g., Horellou et al., J. Physiol., 85, pp. 158-70 (1991). RIN cells are
25 known to endogenously produce GABA and β -endorphin.

Some of the characteristics of various contemplated cells are shown in Table 1.

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Table 1

	<u>Cells</u>	<u>Analgesic Substances</u>	<u>Other Components</u>
	Chromaffin	NE, met-enkephalin	TH, DDC, D β H, PC
	PC12, PC12a	low NE & met-enkephalin	DDC, D β H, PC
5	AtT-20	β -endorphin	DDC, PC
	RINa	β -endorphin, GABA	DDC, PC
	RINb	β -endorphin	DDC, PC
	Neuro 2A		DDC, D β H, PC
	TH =	Tyrosine hydroxylase converts tyrosine - l-dopa	
10	DDC =	Dopamine decarboxylase converts l-dopa - dopamine (DA)	
	D β H =	Dopamine β -Hydroxylase converts DA - norepinephrine (NE)	
	PC =	Prohormone Convertases process POMC to β -endorphin and Pro-enkephalin A (ProA) to met-enkephalin.	
	AtT20 =	Mouse pituitary corticotroph cell line that endogenously secretes β -endorphin	
15	RIN =	Rat insulinoma	
	Neuro 2A =	Mouse neuroblastoma	

The primary delivery products include at least one each of an endorphin, an enkephalin and a catecholamine.

Enkephalins and endorphins are endogenous opioid peptides in humans. These opioid peptides comprise approximately 15 compounds ranging from 5 to 31 amino acids. These compounds bind to and act at least in part via the same μ opioid receptor as morphine, but are chemically unrelated to morphine. In addition, these compounds stimulate other opiate receptors. Yaksh and Malmberg, Textbook of Pain, 3rd Ed. (Eds. P. Wall and R. Melzack), "Central Pharmacology of Nociceptive Transmission," pp. 165-200, 1994 (New York).

The opioid peptides have common chemical properties, but are synthesized in different pathways.

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β -endorphin, the most abundant endorphin, is synthesized as part of a larger precursor molecule, pro-opiomelanocortin ("POMC"). The POMC molecule contains the full sequence of adrenocorticotrophic hormone ("ACTH"), α -melanocyte-stimulating hormone (" α -MSH"), β -MSH, and β -lipotropin. The POMC precursor molecule also has the potential to generate other endorphins, including α -endorphin and gamma-endorphin. Processing of the POMC precursor occurs differently within various tissues according to the localization of cleavage enzymes, such as prohormone convertases, within those tissues.

In the pituitary, POMC is cleaved to produce ACTH and β -endorphin, and the ACTH is not further processed. In contrast, in the hypothalamus, ACTH is converted to β -MSH. While different cell types may synthesize the same primary gene product, the final profile of hormone secretion may differ widely.

This invention contemplates use of a DNA sequence encoding any suitable endorphin that has analgesic activity. In addition, analogs or fragments of these endorphins that have analgesic activity are also contemplated. Thus the endorphin to be produced by the cells of this invention may be characterized by amino acid insertions, deletions, substitutions and modifications at one or more sites in the naturally occurring amino acid sequence of the desired endorphin. We prefer conservative modifications and substitutions (i.e., those having a minimal effect on the secondary or tertiary structure of the endorphin and on the analgesic properties of the endorphin). Such conservative substitutions include those described by

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Dayhoff in Atlas of Protein Sequence and Structure, 5, (1978) and by Argos, Embo J., 3, pp. 779-85 (1989).

Techniques for generating such variants of naturally occurring endorphins are well known. For example, codons in the DNA sequence encoding the wild type endorphin may be altered by site specific mutagenesis.

This invention contemplates using a DNA sequence encoding the entire POMC precursor molecule. This embodiment takes advantage of the host cell's cleavage enzymes (i.e., Prohormone convertase 2) to generate a suite of endorphins, some or all of which may have analgesic properties.

This invention also contemplates use of DNA fragments of the POMC gene that encode a particular desired endorphin.

The DNA and amino acid sequence of POMC are well known. Cochet et al., Nature, 297, pp. 335-9 (1982); Takahashi et al., Nucl. Acids Res., 11, pp. 6847-58 (1983).

We prefer a DNA sequence encoding POMC in which the ACTH coding region has been deleted. The preferred endorphin encoded by this construct is β -endorphin.

Some enkephalins are synthesized in the adrenal glands as part of a large protein, pro-enkephalin A, that contains six repeats of the Met-enkephalin sequence and one Leu-enkephalin structure. Met-enkephalin, as well as Met-enkephalin-Arg-Phe and Met-enkephalin-Arg-Gly-Leu have significant antinociceptive activity. See, e.g., Sagen et al., Brain Res., 502, pp. 1-10 (1989).

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Other enkephalins, i.e., dynorphins and neo-endorphins are derived from a distinct molecule, pro-enkephalin B. Additional "cryptic" peptides are also encoded within the structure of these precursor
5 proteins, and may be released by "pro-hormone-type" cleavage. See, e.g., Harrison's "Principles Of Internal Medicine", 12th Edition, pp. 1168-69 (1991).

This invention contemplates use of a DNA sequence encoding any suitable enkephalin that has
10 analgesic activity. Analogs and active fragments that have analgesic properties are also contemplated. Such analogs or fragments may thus have amino acid insertions, deletions, substitutions at one or more sites in the naturally occurring amino acid sequence.
15 Such variants may be generated as described above.

This invention contemplates use of a DNA sequence encoding a desired enkephalin in its "mature" form. In addition, this invention contemplates using a DNA sequence encoding the entire pro-enkephalin A
20 precursor, or the entire pro-enkephalin B precursor. Further, we also contemplate using DNA encoding a fusion, or fragment of these sequences, that upon expression yields one or more enkephalin-like molecules that have analgesic properties.

25 We prefer use of a DNA sequence encoding the entire pro-enkephalin A precursor molecule. The DNA and amino acid sequence of pro-enkephalin A are well known. Folkesson, supra. This embodiment takes advantage of the host cell's cleavage enzymes, such as
30 prohormone convertase, to generate a suite of enkephalins, some or all of which may have analgesic

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properties. The preferred enkephalin encoded by this construct is Met-enkephalin.

There are three naturally occurring catecholamines which function as neurotransmitters in the central nervous system; norepinephrine ("NE"), epinephrine ("E"), and dopamine. NE is associated with postganglionic sympathetic nerve endings. NE exerts its effects locally in the immediate vicinity of its release.

Catecholamines are synthesized from the amino acid tyrosine, which is sequentially hydroxylated to form dihydroxyphenylalanine (dopa), decarboxylated to form dopamine, and then hydroxylated on the beta position of the side chain by dopamine beta hydroxylase to form NE. Harrison's, supra, pp. 380. NE is N-methylated to E by phenylethanolamine-N methyltransferase ("PNMT").

Hydroxylation of tyrosine by tyrosine hydroxylase ("TH") is the rate limiting step in NE synthesis. Regulation of dopa and NE synthesis in the adrenal medulla may be accomplished by changes in the amount and the activity of TH.

In addition, regulation of synthesis of E from NE may occur by changes in the amount and the activity of phenylethanolamine-N-methyltransferase ("PNMT"). PNMT is inducible by glucocorticoids from the adrenal cortex. Ibid.

Catecholamines are maintained in high concentration in adrenal medullary chromaffin tissue, mostly as E. Opioid peptides are also stored in the adrenal gland.

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NE and E have similar affinities at α_2 receptors and therefore both potentially contribute to analgesia. Bylund, EASEB J., 6, PP. 832-39 (1992). The enkephalin peptides that predominantly include met-enkephalin selectively activate delta (δ) opioid receptors. Reisine and Bell, Trends Neurosci., 16, pp. 506-10 (1993). Activation of α_2 adrenergic and δ opioid receptors in the spinal cord each result in antinociception and are potentially synergistic. Yaksh and Malmberg, Progress in Pain Research and Management, Vol. 1, Ed. Fields and Lisbeskind, IASP Press, Seattle, pp. 141-71 (1994). Activation of δ versus (μ) opioid receptors in experimental animals results in fewer adverse side effects including constipation and addiction liability (Lee et al., J. Pharmacol. Exp. Ther., 267, pp. 883-87 (1993). The combined delivery of different opioidergic and adrenergic agents may decrease the magnitude of tolerance that develops to a single agent and lead to sustained pain relief. Yaksh and Reddy, Anesthesiol., 54, pp. 451-67 (1981).

This invention contemplates use of a DNA sequence encoding catecholamine biosynthetic enzymes or analogs or fragments thereof to obtain catecholamines that have analgesic properties. The preferred catecholamines in this invention are NE and E.

In one embodiment, the host cell is transformed with the genes necessary to accomplish production of NE or E, as desired. The selection of heterologous gene sequences required depends upon the complement of catecholamine synthesizing enzymes normally occurring in the host cell. For example, RIN cells, and AtT-20 cells lack tyrosine hydroxylase

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("TH") and dopamine beta hydroxylase ("DBH"). However, RIN and AtT-20 cells contain endogenous dopa decarboxylase ("DDC"). If the desired catecholamine is E, then the gene encoding PNMT is also required. The
5 gene encoding PNMT is known. Baetge et al., Proc. Nat'l Acad. Sci., 83, pp. 5455-58 (1986).

The gene encoding TH is known. See, e.g., United States patent 5,300,436, incorporated herein by reference. Modified TH variants are also known.
10 United States patent 5,300,436. In addition, truncated versions of TH that contain the necessary C-terminal catalytic domains are also known. See, e.g., Daubner et al., Protein Science, 2, pp. 1452-60 (1993).

AtT-20 cells have been transformed with wild
15 type TH, as well as various TH muteins. See, e.g., Wu et al., J. Biol. Chem., 267, pp. 25754-758 (1992).

The sequence of the DBH gene is also well known. See, e.g., Lamoroux et al., EMBO J., 6, pp. 3931-37 (1987).

20 It will be appreciated that in addition to the preferred DNA sequences described herein, there will be many degenerate DNA sequences that code for the desired analgesics.

Secondary compounds with potential analgesic
25 action may also be produced by the cells of this invention. Such compounds include galanin and somatostatin. In addition, neuropeptide Y, neurotensin and cholecystokinin may be produced by the transformed cells of this invention. The cells of this invention
30 may normally produce some or all of these compounds, or may be genetically engineered to do so using standard techniques.

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Standard methods may be used to obtain or synthesize the genes encoding the analgesic compounds to be produced by the cells of this invention.

For example, the complete amino acid sequence
5 of the desired compound may be used to construct a back-translated gene. A DNA oligomer containing a nucleotide sequence coding for the desired analgesic compound may be synthesized. For example, several small oligonucleotides coding for portions of each
10 desired polypeptide may be synthesized and then ligated. The individual oligonucleotides typically contain 5' or 3' overhangs for assembly.

The DNA sequence encoding each desired analgesic compound, may or may not also include DNA
15 sequences that encode a signal sequence. Such signal sequence, if present, should be one recognized by the cell chosen for expression of the analgesic compound. It may be prokaryotic, eukaryotic or a combination of the two. It may also be the signal sequence of the
20 native compound. It generally is preferred that a signal sequence be encoded and most preferably that the native signal sequence be used.

Once assembled, the DNA sequences encoding the desired compounds will be inserted into one or more
25 expression vectors and operatively linked to expression control sequences appropriate for expression in the desired transformed cell.

Proper assembly may be confirmed by nucleotide sequencing, restriction mapping, and
30 expression of a biologically active polypeptide in the transformed cell. As is well known in the art, in order to obtain high expression levels of a transfected

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gene in a host, the gene must be operatively linked to transcriptional and translational expression control sequences that are functional in the chosen expression cell.

5 The choice of expression control sequence and expression vector will depend upon the choice of cell. A wide variety of expression host/vector combinations may be employed. Useful expression vectors for eukaryotic hosts, include, for example, vectors
10 comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus.

We prefer pcDNA3, pCEP4, pZeoSV (InVitrogen, San Diego) and pNUT.

Any of a wide variety of expression control
15 sequences may be used in these vectors. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors. Examples of useful expression control sequences include, for example, the
20 early and late promoters of SV40 or adenovirus, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating system and other sequences known to control the expression of
25 genes of eukaryotic cells or their viruses, and various combinations thereof.

It should of course be understood that not all vectors and expression control sequences will function equally well to express the DNA sequences
30 described herein. Neither will all cells function equally well with the same expression system. However, one of skill in the art may make a selection among

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these vectors, expression control sequences and cells without undue experimentation. For example, in selecting a vector, the host cell must be considered because the vector must replicate in it. The vector's
5 copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

In selecting an expression control sequence,
10 a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the actual DNA sequence encoding the desired analgesic compounds, particularly as regards potential
15 secondary structures. Host cells should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the DNA sequences, their secretion characteristics, their ability to fold the polypeptides correctly, and their
20 culture requirements. If the host cell is to be encapsulated, cell viability when encapsulated and implanted in a recipient should also be considered.

Within these parameters, one of skill in the art may select various vector/expression control
25 sequence/host combinations that will express the desired DNA sequences in culture.

In one embodiment, cells (e.g., RIN cells) are sequentially transformed with 4 separate expression vectors containing the POMC gene, the pro-enkephalin A
30 gene, the TH gene and the DBH gene. In such a transformed host cell, amplification of copy number of the heterologous genes is more difficult to achieve.

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Thus use of fewer expression vectors is preferred. Most preferably, a single expression vector, containing all 4 heterologous genes, is used.

In a particular embodiment RIN cells are sequentially transformed with 3 expression vectors. The first vector contains the POMC gene operably linked to the CMV promoter. Preferably a truncated version of the POMC gene is used, having the ACTH coding region deleted. The second vector contains the pro-enkephalin A gene operably linked to the CMV promoter. Preferably the proA construct contains the Kozak sequence immediately upstream of the start codon. The third vector contains both the TH gene (preferably truncated and having the Kozak consensus sequence immediately upstream of the start codon) and the DBH gene. In this embodiment, the TH gene is operably linked to the CMV promoter. The DBH gene is operably linked to an internal ribosome entry site promoter sequence. RIN cells are then transformed sequentially with each expression vector according to known protocols.

In another embodiment, a single expression vector containing the pro-enkephalin A gene, the POMC gene, the TH gene, and the DBH gene is constructed. Preferably, the ACTH region of the POMC gene is deleted. Preferably the TH gene is truncated.

Multiple gene expression from a single transcript is preferred over expression from multiple transcription units. One approach for achieving expression of multiple genes from a single eukaryotic transcript takes advantage of sequences in picorna viral mRNAs known as internal ribosome entry sites ("IRES"). These sites function to facilitate protein

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translation from sequences located downstream from the first AUG of the mRNA.

Macejak and Sarnow reported that the 5' untranslated sequence of the immunoglobulin heavy chain binding protein (BiP, also known as CRP 78, the glucose-regulated protein of molecular weight 78,000) mRNA can directly confer internal ribosome binding to an mRNA in mammalian cells, in a 5'-cap independent manner, indicating that translation initiation by an internal ribosome binding mechanism is used by this cellular mRNA. Nature 353, pp. 90-94 (1991).

WO 94/24870 refers to use of more than two IRES for translation initiation from a single transcript, as well as to use of multiple copies of the same IRES in a single construct.

This invention also contemplates use of a "suicide" gene in the transformed cells. Most preferably, the cell carries the TK (thymidine kinase) gene as a safety measure, permitting the host cell to be killed in vivo by treatment with gancyclovir.

Use of a "suicide" gene is known in the art. See, e.g., Anderson, published PCT application WO 93/10218; Hamre, published PCT application WO 93/02556. The recipient's own immune system provides a first level of protection from adverse reactions to the implanted cells. If encapsulated, the polymer capsule itself may be immuno-isolatory. The presence of the TK gene (or other suicide gene) in the expression construct adds an additional level of safety to the recipient of the implanted cells.

Preferred vectors for use in this invention include those that allow the DNA encoding the analgesic

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compounds to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, United States Patent 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) or glutamine synthetase ("GS") amplification (see, e.g., United States patent 5,122,464 and European published application 338,841). Such amplification can be used to increase output of the desired analgesic compounds.

Other techniques for increasing the output of the desired analgesic compounds are contemplated. For example, subcloning existing polyclonal cell lines is contemplated. Cells are cloned by limiting dilution to a single cell in each well. Cell clones are cultures, and the clones are tested to select the clone with the highest output of analgesic substances.

Another technique for increasing the output of the desired analgesic compounds involves cloning altered forms of biosynthetic enzymes with higher activity than the wild type form (i.e., the truncated TH 1-155). Some truncated forms of TH have 4-6 times increased activity over the wild type form of TH. See, e.g., Daubner et al., "Expression and characterization of catalytic and regulatory domains of rat tyrosine hydroxylase" Protein Science, 2, pp. 1452-60 (1993).

In addition, use of tyrosine-free media to select to increase tetrahydrobiopterin cofactor levels may potentially increase tyrosine hydroxylase activity. See, e.g., Horellou et al., "Retroviral transfer of a

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human tyrosine hydroxylase cDNA in various cell lines; regulated release of dopamine in mouse anterior pituitary AtT-20 cells", Proc. Natl. Acad. Sci. USA, 86, pp. 7233-37 (1989).

5 Preferably, the output of β -endorphin ranges between 1 and 10,000 pg/ 10^6 cells/hr. Preferably, the output of met-enkephalin ranges between 1 and 10,000 pg/ 10^6 cells/hr. Preferably, the output of catecholamines ranges between 1 and 1,000 pmoles/ 10^6
10 cells/hr.

The cells of this invention may be implanted into a mammal, including a human, for the treatment of pain. If implanted unencapsulated, any suitable implantation protocol may be used, including those
15 outlined by Sagen et al., United States patent 4,753,635, incorporated herein by reference.

It may be desirable to encapsulate the genetically modified cells of this invention before implantation. Such encapsulated cells form a
20 bioartificial organ ("BAO"). BAOs may be designed for implantation in a recipient or can be made to function extra-corporeally. The BAOs useful in this invention typically have at least one semipermeable outer surface membrane or jacket surrounding a cell-containing core.
25 The jacket permits the diffusion of nutrients, biologically active molecules and other selected products through the BAO. The BAO is biocompatible.

In some cases, the membrane may serve to also immunoisolate the cells by blocking the cellular and
30 molecular effectors of immunological rejection. The use of immunoisulatory membranes allows for the implantation of allo and xenogeneic cells into an

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individual without the use of immunosuppression. If biologically active molecules are released from the isolated cells, they pass through the surrounding semipermeable membrane into the recipient's body. If
5 metabolic functions are provided by the isolated cells, the substances to be metabolized enter the BAO from the recipient's body through the membrane to be acted on by the cells.

A variety of types of membranes have been
10 used in the construction of BAOs. Generally, the membranes used in BAOs are either microporous or ultrafiltration grade membranes. A variety of membrane materials have been suggested for use in BAOs, including PAN/PVC, polyurethanes, polysulfones,
15 polyvinylidienes, and polystyrenes. Typical membrane geometries include flat sheets, which may be fabricated into "sandwich" type constructions, having a layer of living cells positioned between two essentially planar membranes with seals formed around the perimeter of the
20 device. Alternatively, hollow fiber devices may be used, where the living cells are located in the interior of a tubular membrane. Hollow fiber BAOs may be formed step-wise by loading living cells in the lumen of the hollow fiber and providing seals on the
25 ends of the fiber. Hollow fiber BAOs may also be formed by a coextrusion process, where living cells are coextruded with a polymeric solution which forms a membrane around the cells.

BAOs have been described, for example, in
30 United States patent Nos. 4,892,538, 5,106,627, 5,156,844, 5,158,881, and 5,182,111, and PCT Application Nos. PCT/US/94/07015, WO 92/19195, WO

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93/03901, and WO 91/00119, all of which are incorporated herein by reference.

BAOs may contain other components that promote long term survival of the encapsulated cells.

- 5 For example, WO 92/19195 refers to implantable immunoisulatory biocompatible vehicles having a hydrogel matrix for enhancing cell viability.

- The encapsulating membrane of the BAO may be made of a material which is the same as that of the
10 core, or it may be made of a different material. In either case, a surrounding or peripheral membrane region of the BAO which is permselective and biocompatible will be formed. The membrane may also be constructed to be immunoisulatory, if desired. The
15 core contains isolated cells, either suspended in a liquid medium or immobilized within a hydrogel matrix.

- The choice of materials used to construct the BAO is determined by a number of factors and is described in detail in Dionne WO 92/19195. Briefly,
20 various polymers and polymer blends can be used to manufacture the capsule jacket. Polymeric membranes forming the BAO and the growth surfaces therein may include polyacrylates (including acrylic copolymers), polyvinylidenes, polyvinyl chloride copolymers,
25 polyurethanes, polystyrenes, polyamides, cellulose acetates, cellulose nitrates, polysulfones, polyphosphazenes, polyacrylonitriles, poly(acrylonitrile/covinyl chloride), as well as derivatives, copolymers and mixtures thereof.

- 30 BAOs may be formed by any suitable method known in the art. One such method involves coextrusion of a polymeric casting solution and a coagulant which

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can include biological tissue fragments, organelles, or suspensions of cells and/or other therapeutic agents, as described in Dionne, WO 92/19195 and United States Patents 5,158,881, 5,283,187 and 5,284,761, incorporated herein by reference.

The jacket may have a single skin or a double skin. A single-skinned hollow fiber may be produced by quenching only one of the surfaces of the polymer solution as it is co-extruded. A double-skinned hollow fiber may be produced by quenching both surfaces of the polymer solution as it is co-extruded.

Numerous capsule configurations, such as cylindrical, disk-shaped or spherical are possible.

The jacket of the BAO will have a pore size that determines the nominal molecular weight cut off (nMWCO) of the permselective membrane. Molecules larger than the nMWCO are physically impeded from traversing the membrane. Nominal molecular weight cut off is defined as 90% rejection under convective conditions. In situations where it is desirable that the BAO is immunoisulatory, the membrane pore size is chosen to permit the particular factors being produced by the cells to diffuse out of the vehicle, but to exclude the entry of host immune response factors into the BAO. Typically the nMWCO ranges between 50 and 200 kD, preferably between 90 and 150 kD. The most suitable membrane composition will also minimize reactivity between host immune effector molecules known to be present at the selected implantation site, and the BAO's outer membrane components.

The core of the BAO is constructed to provide a suitable local environment for the particular cells

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isolated therein. The core can comprise a liquid medium sufficient to maintain cell growth. Liquid cores are particularly suitable for maintaining transformed cell lines like PC12 cells. Alternatively, the core can comprise a gel matrix. The gel matrix may be composed of hydrogel (alginate, "Vitrogen™", etc.) or extracellular matrix components. See, e.g., Dionne WO 92/19195.

Compositions that form hydrogels fall into three general classes. The first class carries a net negative charge (e.g., alginate). The second class carries a net positive charge (e.g., collagen and laminin). Examples of commercially available extracellular matrix components include Matrigel™ and Vitrogen™. The third class is net neutral in charge (e.g., highly crosslinked polyethylene oxide, or polyvinylalcohol).

Any suitable method of sealing the BAO may be used, including the employment of polymer adhesives and/or crimping, knotting and heat sealing. These sealing techniques are known in the art. In addition, any suitable "dry" sealing method can also be used. In such methods, a substantially non-porous fitting is provided through which the cell-containing solution is introduced. Subsequent to filling, the BAO is sealed. Such a method is described in copending United States application Serial No. 08/082,407, herein incorporated by reference.

One or more in vitro assays are preferably used to establish functionality of the BAO prior to implantation in vivo. Assays or diagnostic tests well known in the art can be used for these purposes. See,

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e.g., Methods In Enzymology, Abelson [Ed], Academic Press, 1993. For example, an ELISA (enzyme-linked immunosorbent assay), chromatographic or enzymatic assay, or bioassay specific for the secreted product
5 can be used. If desired, secretory function of an implant can be monitored over time by collecting appropriate samples (e.g., serum) from the recipient and assaying them. If the recipient is a primate, microdialysis may be used.

10 The number of BAOs and BAO size should be sufficient to produce a therapeutic effect upon implantation is determined by the amount of biological activity required for the particular application. In the case of secretory cells releasing therapeutic
15 substances, standard dosage considerations and criteria known to the art are used to determine the amount of secretory substance required. Factors to be considered are discussed in Dionne, WO 92/19195.

Implantation of the BAO is performed under
20 sterile conditions. Generally, the BAO is implanted at a site in the host which will allow appropriate delivery of the secreted product or function to the host and of nutrients to the encapsulated cells or tissue, and will also allow access to the BAO for
25 retrieval and/or replacement. The preferred host is a primate, most preferably a human.

A number of different implantation sites are contemplated. These implantation sites include the central nervous system, including the brain, spinal
30 cord, and aqueous and vitreous humors of the eye. Preferred sites in the brain include the striatum, the cerebral cortex, subthalamic nuclei and nucleus Basalis

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of Meynert. Other preferred sites are the cerebrospinal fluid, most preferably the subarachnoid space and the lateral ventricles. This invention also contemplates implantation into the kidney subcapsular site, and intraperitoneal and subcutaneous sites, or
5 any other therapeutically beneficial site.

In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only,
10 and are not to be construed as limiting the scope of this invention in any manner.

Examples

Construction of Polycistronic Expression Vectors

Construction of IgSP-POMC Fusion

15 The SmaI-SalI fragment containing the human POMC exon 3 was subcloned into pBS cloning vector (Stratagene). See Takahashi, supra; Cochet, supra. The resulting plasmid was named as pBS-hPOMC-027. See Fig. 1.

20 A PCR fragment was generated using two oligonucleotide primers, termed oCNTF-003 (SEQ ID NO: 1) and oIgSP-018, (SEQ ID NO: 2) and the pNUT plasmid containing the human CNTF gene. See Baetge et al., Proc. Natl. Acad. Sci. USA, 83, pp. 5454-58
25 (1986). Both primers oCNTF-003 and oIgSP-018, contain synthetic BamHI and SmaI restriction sites, respectively, at the 5' ends.

The 196 base pair (bp) PCR fragment was digested with restriction endonucleases BamHI and the
30 SmaI-isoschizomer XmaI, and electrophoresed through an

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1% SeaPlaque agarose. The 193 bp HindIII/XmaI DNA fragment was excised and purified using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME).

5 pBS-hPOMC-027 was also digested with BamHI and XmaI and purified from 1% SeaPlaque agarose using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME). The ligation mixture was transformed into E. coli DH5 α (Gibco BRL, Gaithersburg, MD).

10 Positive sub-clones were initially identified by the cracking gel procedure (Promega Protocols and Applications Guide, 1991). Minilysate DNA was then prepared using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME) and subject to BamHI
15 and SmaI restriction digestions. The positive sub-clone was named as pBS-IgSP-hPOMC-028. See Fig. 1. The nucleotide sequence of the fusion junction in pBS-IgSP-hPOMC-028 was determined by the dideoxynucleotide sequence determination using the Sequenase kit (USBC,
20 Cleveland). The sequence of the IgSP-hPOMC fusion is shown in SEQ ID NO: 3.

Construction of IgSP-POMC Expression Vectors

The IgSP-hPOMC DNA fragment in pBS-IgSP-hPOMC-028 was subcloned into pcDNA3 (Invitrogen Corp.,
25 San Diego, CA) and pCEP4 (Invitrogen Corp., San Diego, CA) in sense and anti-sense orientations.

The NotI-SalI IgSP-hPOMC fragment from pBS-IgSP-hPOMC-028 was ligated with the NotI-XhoI digested pCEP4 resulting in the sense orientation clone named as
30 pCEP4-hPOMC-030. Fig. 2. The BamHI-SalI IgSP-hPOMC fragment from pBS-IgSP-hPOMC-028 was ligated with the

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BamHI-XhoI digested pCEP4 resulting in the anti-sense orientation clone named as pCEP4-hPOMC-031. Fig. 2. The insert orientation in pCEP4-hPOMC-030 and -031 was confirmed by BamHI, NotI, SalI and NotI/SalI restriction digestions as well as by dideoxynucleotide sequence determination using the Sequenase kit (USBC, Cleveland).

The BamHI-SalI IgSP-hPOMC fragment from pBS-IgSP-hPOMC-028 was ligated with the BamHI-XhoI digested pcDNA3 resulting in the sense orientation clone named as pcDNA3-hPOMC-034. Fig. 2. The NotI-HindIII IgSP-hPOMC fragment from pBS-IgSP-hPOMC-028 was ligated with the NotI-HindIII digested pcDNA3 resulting in the anti-sense orientation clone named as pcDNA3-hPOMC-035. Fig. 2. Restriction digestion using SmaI, BamHI, EcoRI, and BamHI/EcoRI was used to confirm the insert orientation in pcDNA3-hPOMC-034, whereas HindIII, NotI and SalI were used for pcDNA3-hPOMC-035.

Construction of ACTH Deleted IgSP-POMC

The ACTH coding region in the POMC gene in pBS-IgSP-hPOMC-028 was deleted. pBS-IgSP-hPOMC-028 was first digested with XmaI restriction enzyme and treated with pfu DNA polymerase (Promega, Madison, WI). The XmaI-pfu DNA polymerase treated pBS-IgSP-hPOMC-028 was then digested with StuI restriction enzyme and purified from 1% SeaPlaque agarose using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME). The self-ligation mixture was transformed into E. coli DH5 α (Gibco BRL, Gaithersburg, MD). Positive sub-clones were identified by BamHI/HindIII restriction digestion and named as pBS-IgSP-hPOMC Δ ACTH-029. See Fig. 1. The

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nucleotide sequence of the ACTH deletion region in pBS-IgSP-hPOMC- Δ ACTH-029 was confirmed by the dideoxynucleotide sequence determination. The sequence of the IgSP-hPOMC- Δ ACTH fusion is shown in SEQ ID

5 NO: 4.

Construction of ACTH Deleted IgSP-POMC Expression Vectors

The IgSP-hPOMC- Δ ACTH DNA fragment in pBS-IgSP-hPOMC- Δ ACTH-029 was subcloned into pcDNA3
10 (Invitrogen Corp., San Diego, CA) and pCEP4 (Invitrogen Corp., San Diego, CA) in sense and anti-sense orientations. The NotI-SalI IgSP-hPOMC- Δ ACTH fragment from pBS-IgSP-hPOMC- Δ ACTH-029 was ligated with the NotI-XhoI digested pCEP4 resulting in the sense
15 orientation clone named as pCEP4-hPOMC- Δ ACTH-032 (Fig. 3). The BamHI-SalI IgSP-hPOMC- Δ ACTH fragment from pBS-IgSP-hPOMC- Δ ACTH-029 was ligated with the BamHI-XhoI digested pCEP4 resulting in the anti-sense orientation clone named as pCEP4-hPOMC- Δ ACTH-033
20 (Fig. 3). The insert orientation in pCEP4-hPOMC- Δ ACTH-032 and -033 was confirmed by BamHI and EcoRI restriction digestions as well as by dideoxynucleotide sequence determination using the Sequenase kit (USBC, Cleveland).

25 The BamHI-SalI IgSP-hPOMC- Δ ACTH fragment from pBS-IgSP-hPOMC- Δ ACTH-029 was ligated with the BamHI-XhoI digested pcDNA3 resulting in the sense orientation clone named as pcDNA3-hPOMC- Δ ACTH-036 (Fig. 3). The NotI-HindIII IgSP-hPOMC- Δ ACTH fragment from pBS-IgSP-
30 hPOMC- Δ ACTH-029 was ligated with the NotI-HindIII

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digested pcDNA3 resulting in the anti-sense orientation clone named as pcDNA3-hPOMC- Δ ACTH-037 (Fig. 3).

Restriction digestion using PvuII and EcoRI was used to confirm the insert orientation in pcDNA3-hPOMC- Δ ACTH-036, whereas SalI and EcoRI were used for pcDNA3-hPOMC- Δ ACTH-037.

Cloning of Full Length and Truncated TH cDNA

Total RNA from PC12 cells was prepared using the guanidinium thiocyanate-based TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). Five hundred ng of PC12 total RNA was reverse transcribed at 42°C for 30 minutes in a 20 μ l reaction volume containing 10 mM Tris.HCl (pH 8.3), 50 mM KCl, 4 mM of each dNTP, 5 mM MgCl₂, 1.25 μ M oligo (dT) 15-mer, 1.25 μ M random hexamers, 31 units of RNase Guard RNase Inhibitor (Pharmacia, Sweden) and 200 units of SuperScript II reverse transcriptase (Gibco BRL, Gaithersburg, MD). Two micro-liters of the above reverse transcribed cDNA was added to a 25 μ l PCR reaction mixture containing 10 mM Tris.HCl (pH 8.3), 50 mM KCl, 800 of each nM dNTP, 2 mM MgCl₂, 400 nM of primers #1 and #2, and 2.5 units of Thermus aquaticus (Taq) DNA polymerase (Boehringer Mannheim, Germany).

To generate the full length TH cDNA, oligonucleotide primers orTH-052 (SEQ ID NO: 5) and orTH-053 (SEQ ID NO: 6) were used. For the truncated TH, primers orTH-054 (SEQ ID NO: 7) and orTH-053 (SEQ ID NO: 6) were used instead. These oligonucleotides were constructed based on published TH sequence information in Grima et al., Nature, 326, pp. 707-11 (1987); US patent 5,300,436, and Daubner, supra.

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Primers orTH-052 (SEQ ID NO: 5) and orTH-054 (SEQ ID NO: 7) have synthetic HindIII restriction site at the 5' end where orTH-053 has BamHI at the 5' end. The PCR reaction mixtures were subject to 30
5 amplification cycles consisted of: denaturation, 94°C 30 seconds (first cycle 2 minutes); annealing, 50°C 1 minute; and extension, 72°C 3.5 minutes (last cycle 5 minutes). The 1537 bp full length and 1087 bp truncated rat TH PCR fragments were digested with
10 restriction endonucleases BamHI and HindIII and resolved on an 1% SeaPlaque agarose gel. The 1531-bp and 1081-bp HindIII/BamHI DNA fragments were excised and purified using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME).

15 pcDNA3 expression vector was also digested with BamHI and HindIII and purified from 1% SeaPlaque agarose using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME). The ligation mixture was transformed into E.coli DH5 α (Gibco BRL,
20 Gaithersburg, MD).

Cracking gel procedure (Promega Protocols and Applications Guide, 1991) was used to screen out the positive sub-clones. The identity of the correct clones was further verified by BamHI/HindIII double
25 digestion.

The positive sub-clones for the full-length and truncated rat TH in pcDNA3 were named as pcDNA3-rTH-044 (Fig. 4) and pcDNA3-rTH Δ -045 (Fig. 4), respectively. The nucleotide sequence of both full-
30 length and truncated rat TH PCR clones was determined by the dideoxynucleotide sequence determination using

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the Sequenase kit (USBC, Cleveland). The sequence of the rTHA construct is shown in SEQ ID NO: 16.

To optimize the translation efficiency of the truncated rat TH, oligonucleotide primer orTH-078 (SEQ ID NO: 8) was designed so that the consensus Kozak sequence is immediate up stream to the start codon ATG. pcDNA3-rTHA-45 was used as the template in a 50 µl PCR reaction mixture with reagent composition identical to the one described above with the exception that the oligonucleotide primers were replaced with orTH-078 (SEQ ID NO: 8) and orTH-053 (SEQ ID NO: 6). The 1097 bp PCR product was cloned into pcDNA3 in the same manner as described above. The resulting sub-clone was named pcDNA3-rTHAKS-75 (Fig 4). The sequence of the rTHAKS construct is shown in SEQ ID NO: 17.

Construction of rTH-IRES-bDBH Fusion Gene

Recombinant PCR methodology was used to generate the rTH-IRES-bDBH fusion gene. Oligonucleotides oIRES-057 (SEQ ID NO: 9) and obDBH-065 (SEQ ID NO: 10) are specific for IRES and bDBH gene sequences, respectively, and contain synthetic BamHI and NotI restriction sites at the 5' end, respectively. Oligonucleotides oIRES-bDBH-064 (SEQ ID NO: 11) and oIRES-bDBH-066 (SEQ ID NO: 12) are complementary to each other. Furthermore, oligonucleotide primer oIRES-bDBH-064 (SEQ ID NO: 11) has its 5' 16 nucleotides identical to the IRES sequence and its 3' 18 nucleotides identical to the bDBH sequence; and vice versa for oIRES-bDBH-066 (SEQ ID NO: 12).

Two first PCR reactions were carried out using oligonucleotide pairs oIRES-057/oIRES-bDBH-066

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and oIRES-bDBH-064/obDBH-065 on templates pCTI-001 (with an insert containing the IRES sequence shown in SEQ ID NO: 30) and pBS-bDBH-006 (containing the bovine DBH gene cloned from bovine adrenal chromaffin cells, 5 Lamoroux et al., EMBO J., 6, pp. 3931-37 (1987)) plasmids, respectively. One hundred ng of template DNA was added to a 50 µl PCR reaction mixture containing 10 mM Tris.HCl (pH 8.3), 50 mM KCl, 800 of each nM dNTP, 2 mM MgCl₂, 400 nM of primers #1 and #2, and 2.5 10 units of Thermus aquaticus (Taq) DNA polymerase (Boehringer Mannheim, German).

The PCR reaction mixtures were subject to 30 amplification cycles consisted of: denaturation, 94 °C for 30 seconds (first cycle 2 minutes); annealing, 15 50 °C 1 minute; and extension, 72 °C 30 seconds (last cycle 5 minutes). The PCR products were resolved on 1% TrivieGel 500 (TrivieGen). Two agarose plugs containing each one of the first PCR products were transfer to a tube containing 50 µl of PCR reaction 20 mixtures identical to the one described above with the exception that the oligonucleotides oIRES-057 and obDBH-065 were used.

The second PCR reaction was subject to 30 amplification cycles consisted of: denaturation, 94 °C 25 for 30 seconds (first cycle 2 minutes); annealing, 60 °C 30 seconds (second to fourth cycles 37 °C 2 minutes); and extension, 72 °C 30 seconds (last cycle 2 minutes). The 2407 bp IRES-bDBH fusion PCR product and the cloning vector pcDNA3-rTHA-45 were digested with 30 BamHI and NotI restriction enzymes and subsequently purified from 1% SeaPlaque agarose gel using the FMC

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SpinBind DNA purification kit (FMC BioProducts, Rockland, ME).

The ligation of IRES-bDBH/BamHI/NotI and pcDNA3-rTHA-045/BamHI/NotI would generate a rTHA-IRES-bDBH expression vector named as pcDNA3-rTHA-IRES-bDBH-066 (Fig. 5) whereas that of IRES-bDBH/BamHI/NotI and pcDNA3-rTHAKS-075/BamHI/NotI would generate a rTHAKS-IRES-bDBH expression vector, named as pcDNA3-rTHAKS-IRES-bDBH-076 (Fig. 5), where the start codon ATG in rTHA is preceded with a consensus Kozak sequence. The sequence of the rTHA-IRES-bDBH construct is shown in SEQ ID NO: 18. The sequence of the rTHAKS-IRES-bDBH construct is shown in SEQ ID NO: 19. The ligation mixture was transformed into DH5 α (Gibco BRL, Gaithersburg, MD). The positive clones were identified by the cracking gel procedure (Promega, Madison, WI) and restriction digestions using HindIII, BamHI, HindIII/BamHI, SmaI and NotI.

The 4114 bp NruI-XhoI fragment containing the CMV promoter-rTHAKS-IRES-bDBH was excised out of pcDNA3-rTHAKS-IRES-bDBH-076 and subcloned into pZeoSV cloning vector (Invitrogen Corp., San Diego, CA) digested with ScaI and XhoI in the multiple cloning site. The resulting expression vector was named as pZeo-Pcmv-rTHAKS-IRES-bDBH-088 (Fig. 6).

Construction of IgSP-hPOMC ACTH-rTHD-IRES-bDBH Fusion Gene

The 4100 bp NruI-NotI fragment containing the CMV promoter, rTHD-IRES-bDBH fusion gene, and BGH polyadenylation sequence was excised out of pcDNA3-

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rTHA-IRES-bDBH-066 and subcloned into the pBS (Stratagene, La Jolla, CA) cloning vector.

The resulting plasmid pBS-Pcmv-rTHA-IRES-bDBH-067 (Fig. 7) was used as the intermediary
5 construct to which the recombinant PCR IgSP-hPOMCDACTH-IRES fragment would be inserted.

Oligonucleotide oIgSP-068 (SEQ ID NO: 13), containing a synthetic EcoRV restriction site, is specific for the IgSP sequence.

10 Oligonucleotide primer orTHA-073 (SEQ ID NO: 14) is specific for the rTHA sequence and contains an endogenous SmaI restriction site.

Oligonucleotide primers ohPOMC-IRES-069 (SEQ ID NO: 15) and ohPOMC-IRES-070 (SEQ ID NO: 20) are
15 complementary to each other. Furthermore, oligonucleotide primer ohPOMC-IRES-069 has its 5', 18 nucleotides identical to the hPOMC sequence and its 3' 12 nucleotides identical to the IRES sequence; and vice versa for ohPOMC-IRES-070.

20 Oligonucleotide primers oIRES-rTHA-071 (SEQ ID NO: 21) and oIRES-rTHA-072 (SEQ ID NO: 22) are complementary to each other. In addition, oligonucleotide primer oIRES-rTHA-071 has its 5' 15 nucleotides identical to the rTHA sequence and its 3'
25 18 nucleotide identical to the IRES sequence; and vice versa for oIRES-rTHA-072.

Three sets of first PCR reactions were carried out.

PCR reaction A: template pBS-IgSP-hPOMCDACTH-029,
30 oligonucleotides oTgSP-068/ohPOMC-IRES-069;

PCR reaction B: template pCTI-001,
oligonucleotides ohPOMC-IRES-070/oIRES-rTHA-071; and

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PCR reaction C: template pcDNA3-rTHA-045,
oligonucleotides orIRES-rTHA-072/orTHA-073.

The three sets of first PCR reactions were
carried in 50 µl PCR reaction mixture containing 100 ng
5 of template DNA, 10 mM Tris. HCl (pH 8.3), 50 mM KCl,
800 of each nM dNTP, 2 mM MgCl₂, 400nM of primers #1
and #2, and 2.5 units of Thermus aquaticus (Taq) DNA
polymerase (Boehringer Mannheim, Germany).

The PCR reaction mixtures were subject to 30
10 amplification cycles consisted of: denaturation, 94 °C
for 30 seconds (first cycle 2 minutes); annealing,
50 °C 1 minute; and extension, 72 °C 30 seconds (last
cycle 5 minutes).

The PCR products were resolved on 1%
15 TrivieGel 500 (TrivieGen). Two agarose plugs
containing each one of the PCR products from PCR
reactions B and C were transferred to a tube containing
50 µl of PCR reaction mixtures identical to the one
described above with the exception that the
20 oligonucleotides ohPOMC-IRES-070 and orTHA-073 were
used.

The second PCR reaction was subject to 30
amplification cycles consisted of: denaturation, 94 °C
for 30 seconds (first cycle 2 minutes); annealing,
25 60 °C 30 seconds (second to fourth cycles 37 °C 2
minutes); and extension, 72 °C 30 seconds (last cycle 2
minutes).

The PCR products were treated as described
above. Agarose plugs containing the PCR products from
30 the second PCR reaction and the PCR reaction A were
combined and subjected to a third PCR amplification
using oIgSP-068/rTHA-073. The 1203 bp IgSP-hPOMC-IRES-

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rTHA fusion PCR product and the cloning vector pBS-Pcmv-rTHA-IRES-bDBH-067 were digested with EcoRV and XmaI restriction enzymes and subsequently purified from 1% SeaPlaque agarose gel using the FMC SpinBind DNA
5 purification kit (FMC BioProducts, Rockland, ME). The ligation mixture was transformed into DH5 α (Gibco BRL, Gaithersburg, MD).

The positive clones were identified by the cracking gel procedure (Promega, Madison, WI) and
10 restriction digestions using EcoRI, KpnI and NotI. The resulting clone was named as pBS-IgSP-hPOMCACTH-IRES-rTHA-IRES-bDBH-068. Fig. 8. The sequence of this construct is shown in SEQ ID NO: 23.

15 **Construction of IgSP-hPOMCACTH-IRES-rTHA-IRES-bDBH Expression Vectors**

The 4491 bp NotI fragment containing the IgSP-hPOMCACTH-IRES-rTHA-IRES-bDBH gene was excised out of the pBS-IgSP-hPOMCACTH-IRES-rTHA-IRES-bDBH-068 and subcloned into the pcDNA3 (Invitrogen Corp., San
20 Diego, CA) at the NotI site in the multiple cloning site. Restriction digestion using NotI and SmaI confirmed that the IgSP-hPOMCACTH-IRES-rTHA-IRES-bDBH gene was inserted in the sense orientation resulting in pcDNA3-IgSP-hPOMCACTH-IRES-rTHA-IRES-bDBH-069. See
25 Fig. 9.

Construction of IgSP-hPOMCACTH-IRES-rTHA-IRES-bDBH-IRES-Zeocine Expression Vector

Recombinant PCR methodology was used to generate the IRES-Zeocine fusion gene.
30 Oligonucleotides oIRES-074 (SEQ ID NO: 24) and oZeocin-

- 40 -

077 (SEQ ID NO: 25) are specific for IRES and Zeocin gene sequences, respectively, and contain synthetic NotI and XhoI restriction sites at the 5' end, respectively. Oligonucleotides oIRES-Zeocin-075 (SEQ ID NO: 26) and oIRES-Zeocin-076 (SEQ ID NO: 27) are complementary to each other. Furthermore, oligonucleotide oIRES-Zeocin-075 has its 5'15 nucleotides identical to the Zeocin sequence and its 3' 18 nucleotides identical to the IRES sequence; and vice versa for oIRES-Zeocin-076.

Two first PCR reactions were carried out using oligonucleotide pairs oIRES-074/oIRES-Zeocin-075 and oIRES-Zeocin-076/oZeocin-075 on templates pCTI-001 and pZeoSV (Invitrogen Corp., San Diego, CA) plasmids, respectively.

One hundred ng of template DNA was added to a 50 µl PCR reaction mixture containing 10mM Tris.HCl (pH 8.3), 50 mM KCl, 800 of each nM dNTP, 2 mM MgCl₂, 400 nM of primers #1 and #2, and 2.5 units of Thermus 20 aquaticus (Taq) DNA polymerase (Boehringer Mannheim, Germany).

The PCR reaction mixtures were subject to 30 amplification cycles consisted of: denaturation, 94 °C for 30 seconds (first cycle 2 minutes); annealing, 25 50 °C 1 minute; and extension, 72 °C 30 seconds (last cycle 5 minutes).

The PCR products were resolved on 1% TrivieGel 500 (TrivieGen). Two agarose plugs containing each one of the first PCR products were 30 transfer to a tube containing 50 µl of PCR reaction mixtures identical to the one described above with the

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exception that the oligonucleotides oIRES-074 and oZeocin-077 were used.

The second PCR reaction was subject to 30 amplification cycles consisted of: denaturation, 94 °C for 30 seconds (first cycle 2 minutes); annealing, 50 °C 30 seconds (second to fourth cycles 37 °C 2 minutes); and extension, 72 °C 30 seconds (last cycle 2 minutes).

The 974 bp IRES-Zeocin fusion PCR product and the cloning vector pcDNA3 were digested with NotI and XhoI restriction enzymes and subsequently purified from 1% SeaPlaque agarose gel using the FMC SpinBind DNA purification kit (FMC BioProducts, Rockland, ME).

The ligation of IRES-Zeocin/NotI/XhoI and pcDNA3/NotI/XhoI would generate an intermediate cloning vector named as pcDNA3-IRES-Zeocin-072. Fig. 10.

The positive clones were identified by the cracking gel procedure (Promega, Madison, WI) and restriction digestions using HindIII, SmaI, XhoI, NotI and NotI/XhoI.

To generate the final IgSP-hPOMCΔACTH-IRES-rTHD-IRES-bDBH-IRES-Zeocine Expression Vector, a 4491 bp NotI fragment containing the IgSP-hPOMCΔACTH-IRES-rTHA-IRES-bDBH gene was excised out of the pBS-IgSP-hPOMCΔACTH-IRES-rTHA-IRES-bDBH-068 (Fig. 8; SEQ ID NO: 23) and subcloned in to the pcDNA3-IRES-Zeocin-072 (Fig. 10) at the NotI site in the multiple cloning site.

Restriction digestion using NotI and SmaI confirmed that the IgSP-hPOMCΔACTH-IRES-rTHA-IRES-bDBH gene was inserted in the sense orientation resulting in pcDNA3-IgSP-hPOMCΔACTH-IRES-rTHA-IRES-bDBH-IRES-Zeocin-

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073. The sequence of this construct is shown in SEQ ID NO: 28. Fig. 11.

Construction of ProA+KS Fusion

A construct containing the coding region of the human pro-enkephalin A gene with the consensus Kozak sequence immediately upstream to the start codon ATG. The sequence of this construct is shown in SEQ ID NO: 29.

Construction of hProA+KS Expression Vector

The HindIII/BamHI fragment containing the hProA+KS fusion was ligated into BamHI and Hind III digested pcDNA3 expression vector substantially as described above. After screening as described above, a positive sub-clone was named pcDNA3-hProA+KS-091. Fig. 12. Construction of the pBS-CMV Pro A vector is detailed in Mothis, J. and Lindberg, I., Endocrinology, 131, pp. 2287-96 (1992).

Transformation of Cells

RIN and AtT-20 cells were transformed as follows.

The RINa and AtT-20 based cell lines were grown in DMEM (Gibco) with 10% fetal bovine serum and pen-strep-fungizone (Gibco) base media. The cells were plated out in P100 petri dishes (750,000 cells/dish) in 10 ml of base media. 18-24 hours later, the cells were transfected using calcium phosphate method with a kit made by Stratagene (San Diego, CA). A 10 µg amount of the plasmid vector DNA was diluted in 450 µl of deionized sterile water. Then, 50 µl of a 10x buffer

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(solution #1) was added to the plasmid DNA. A 500 µl amount of solution #2 was immediately added to the DNA containing solution and mixed gently. This was incubated at room temperature for 20 minutes and then
5 the 1.0 ml solution was added to the cells in the petri dish. The cells were incubated overnight and 18-24 hours later the cells were washed 2x with Hanks balanced salt solution without calcium and magnesium. Then, the cells were cultured in base media + selection
10 drugs. The cells were selected in either 600 µg/ml geneticin (Gibco) or 400 µg/ml hygromycin (Boehringer Mannheim) or 500 µg/ml Zeocin (In Vitrogen, San Diego, CA). Cells were sequentially transfected and selected to obtain the final cell line.

15 The RINa cells were transfected with plasmid pCEP4-hPOMC-030 containing the POMC gene. This is a hygromycin resistant vector. The cells were also transformed with plasmid pcDNA3-hProA+KS-091. This is a geneticin resistant vector. Finally, the cells were
20 transfected with plasmid pZeo-PCMV-rTHAKS-IRES-bDBH-088 which conferred Zeocin resistance.

The AtT-20 cells were transfected with plasmid pBS-CMV-ProA and pCEP4-POMC-ΔACTH-32 which conferred geneticin and hygromycin resistance,
25 respectively. Finally, the cells were transfected with plasmid pZeo-Pcmv-rTHAKS-IRES-bDBH-088.

We have tested a number of media for cell growth. Surprisingly we have found that in certain serum-free medias, the above cell lines have enhanced
30 neurotransmitter output, compared to serum-containing media. We prefer CHO-Ultra (Biowhitaker) for the

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growth of AtT-20 cells, and Ultra-Culture (Biowhitaker) for the growth of RINa cells.

Output of various analgesics from one transformed RINa cell line (RINa/ProA/P030/P088) is shown in Table 2. All values represent unstimulated cells. Output of β -endorphin and met-enkephalin is in pg/ 10^6 cells/hr. β -endorphin and met-enkephalin were measured by radioimmunoassay using Incstar kits (Stillwater, Minnesota). Catecholamine output is in pmoles/ 10^6 cells/hr. The numbers in parentheses represent values from cells that were preincubated 18 hours with 100 μ M tetrahydrobiopterin. Catecholamines were measured by high performance liquid chromatography as described in Lavoie et al., "Two PC12 pheochromocytoma lines sealed in hollow fiber-based capsules tonically release l-dopa in vitro", Cell transplantation, 2, pp. 163-73 (1993). GABA output from these RINa cells was 28 ng/ 10^6 cells/hrs.

Table 2

20	<u>Cell Line</u>	<u>Endogenous Analgesic Substances</u>	<u>β-endorphin</u>	<u>Met-enk</u>	<u>DA E</u>
	RIN a/ ProA/ POMC/ 25 TH-IRES-D β H	β -endorphin GABA	22	17	3 0 (6) (2)

There are encrypted enkephalin fragments which are not fully processed from the pro-enkephalin precursor molecule. These encrypted enkephalins have opioid receptor binding activity. We digested these encrypted enkephalins to measure opioid activity. The trypsin digest protocol is as follows. A 2 μ g/ml trypsin (Worthington #34E470) solution is added to media

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samples on ice. Samples are vortexed, then incubated for 20 minutes in a 37°C waterbath. After the 20 minute digest, samples are returned to ice and 100 ng/ml carboxypeptidase B (Sigma #C-7011) is added.

5 Samples are mixed by vortexing, and returned to the 37°C waterbath for 15 minutes. Samples are placed on ice once more and 10 ug/ml trypsin inhibitor is added. At this stage, samples are either extracted for met-enkephalin or immediately frozen for future extraction.

10 This results in the full enzymatic cleavage to free all met-enkephalin from the longer encrypted fragments. A met-enkephalin radioimmunoassay of the digested sample gives total met-enkephalin from the supernatant. The transformed RINa cells appear to have greater than 5

15 fold more encrypted enkephalins compared to fully processed met-enkephalin.

Fiber capsule formation and characteristics

Hollow fibers are spun from a 12.5-13.5% poly(acrylonitrile vinylchloride) solution by a wet

20 spinning technique. Cabasso, Hollow Fiber Membranes, vol. 12, Kirk-Othmer Encyclopedia of Chemical Technology, Wiley, New York, 3rd Ed. pp. 492-517 (1980), United States patent 5,158,881, incorporated herein by reference.

25 The resulting membrane fibers may either be double skinned or single skinned PAN/PVC fibers. In order to make implantable capsules, lengths of fiber are first cut into 5 cm long segments and the distal extremity of each segment sealed with an acrylic glue.

30 Encapsulation hub assemblies are prepared by providing lengths of the membrane described above, sealing one

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end of the fiber with a single drop of LCM 24 (Light curable acrylate glue, available from ICI), curing the glue with blue light, and repeating the step with a second drop. The opposite end is previously attached
5 to a frangible necked hub assembly, having a silicone septum through which the cell solution may be introduced. The fiber is glued to the hub assembly by applying LCM 22 to the outer diameter of the hub assembly, pulling the fiber up over it, and curing with
10 blue light. The hub/fiber assemblies are placed in sterilization bags and are ETO sterilized.

Following sterilization with ethylene oxide and outgassing, the fibers are deglycerinated by ultrafiltering first 70% EtOH, and then HEPES buffered
15 saline solution through the walls of the fiber under vacuum.

Preparation and Encapsulation of Transformed Cells

The transformed cells are prepared and encapsulated as follows:

20 A matrix solution is prepared using a commercially available alginate, collagen or other suitable matrix material. The cell solution was diluted in the ratio of two parts matrix solution to one part cell solution containing the transformed cells
25 described above. We prefer Vitrogen (Celtix, Santa Clara) as a matrix for AtT-20 cells.

We prefer Organogen (Organogenesis, Canton, MA) as a matrix for RINa cells. The RINa based cells are prepared for encapsulation by the following method.
30 The cells are grown in base media of DMEM + 10% fetal bovine serum during the proliferation phase. These

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cells can be removed from the tissue culture flasks by two washes in Hanks balanced salt solution without calcium and magnesium. Then the cells are incubated in 0.25% trypsin + EDTA for 1 minute. This is removed and
5 the cells are rinsed free of the flask using Hanks balanced salt solution without calcium and magnesium solution. The cells are placed in 10 mls of base media and centrifuged at 100 x g for 2 minutes. The cells are resuspended in 10 mls of the preferred serum free
10 media (Ultra culture, Biowhitaker, Walkersville, MD). Surprisingly, the RINa cells secrete more analgesic substances when cultured in this serum free media relative to serum containing base media.

The cells are centrifuged at 100 g twice in
15 the preferred serum free media before the cells are concentrated 1:1 with the preferred Organogen matrix. Organogen is a 1% bovine tendon collagen obtained as a sterile solution. 8 parts of this solution are mixed with 1 part 10X DPBS. 0.5 N sodium hydroxide is added
20 until physiological pH is attained (approximately 250 μ ls).

The final concentration of the cell + matrix solution used for encapsulation can range from 20,000 - 50,000 cells/ μ l. The cells are counted in a standard
25 manner on a hemocytometer.

The cell/matrix suspension is placed in a 1 ml syringe. A Hamilton 1800 Series 50 microliter syringe is set for a 15 microliter air bubble, is inserted into a 1 ml syringe containing the cell
30 solution and 30 microliters are drawn up. The cell solution is injected through the silicone seal of the hub/fiber assembly into the lumen of a modacrylic

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hollow fiber membrane with a molecular weight cutoff of approximately 50,000-100,000 daltons. Ultrafiltration should be observed along the entire length of the fiber. After one minute, the hub is snapped off the sub-hub, exposing a fresh surface, unwet by cell solution. A single drop of LCM 24 is applied and the adhesive cured with blue light. The device is placed first in HEPES buffered NaCl solution and then in CaCl_2 solution for five minutes to cross-link the alginate. Each implant is about 5 cm long, 1 mm in diameter, and contained approximately 2.5 million cells.

After the devices are filled and sealed, a silicone tether (Speciality Silicone Fabrication, Paso Robles, CA) (ID: 0.69, OD: 1.25) is then placed over the proximal end of the fiber. A radiopaque titanium plug is inserted in the lumen of the silicone tether to act as a radiographic marker. The devices are then placed in 100 mm tissue culture dishes in 1.5 ml PC-1 medium, and stored at 37°C, in a 5% CO_2 incubator for in vitro analysis and for storage until implantation.

The encapsulated cells are then implanted into the human sub-arachnoid space as follows:

Surgical Procedure

After establishing IV access and administering prophylactic antibiotics (cefazolin sodium, 1 gram IV), the patient is positioned on the operating table, generally in either the lateral decubitus or genu-pectoral position, with the lumbar spine flexed anteriorly. The operative field is sterily prepared and draped exposing the midline dorsal lumbar region from the levels of S-1 to L-1, and

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allowing for intraoperative imaging of the lumbar spine with C-arm fluoroscopy. Local infiltration with 1.0% lidocaine is used to establish anesthesia of the skin as well as the periosteum and other deep connective tissue structures down to and including the ligamentum flavum.

A 3-5 cm skin incision is made in the parasagittal plane 1-2 cm to the right or left of the midline and is continued down to the lumbodorsal fascia using electrocautery for hemostasis. Using traditional bony landmarks including the iliac crests and the lumbar spinous processes, as well as fluoroscopic guidance, and 18 gauge Touhy needle is introduced into the subarachnoid space between L-3 and L-4 via an oblique paramedian approach. The needle is directed so that it enters the space at a shallow, superiorly directed angle that is no greater than 30-35° with respect to the spinal cord in either the sagittal or transverse plane. Appropriate position of the tip of the needle is confirmed by withdrawal of several ml of cerebrospinal fluid (CSF) for preimplantation catecholamine, enkephalin, glucose, and protein levels and cell counts.

The Touhy needle hub is reexamined to confirm that the opening at the tip is oriented superiorly (opening direction is marked by the indexing notch for the obturator on the needle hub), and the guide wire is passed down the lumen of the needle until it extends 4-5 cm into the subarachnoid space (determined by premeasuring). Care is taken during passage of the wire that there is not resistance to advancement of the wire out of the needle and that the patient does not

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complain of significant neurogenic symptoms, either of which observations might indicate misdirection of the guide wire and possible impending nerve root or spinal cord injury.

5 After the guide wire appears to be appropriately placed in the subarachnoid space, the Touhy needle is separately withdrawn and removed from the wire. The position of the wire in the midline of the spinal canal, anterior to the expected location of
10 the caud equina, and without kinks or unexplainable bends is then confirmed with fluoroscopy. After removal of the Touhy needle the guide wire should be able to be moved freely into and out of the space with only very slight resistance due to the rough surface of
15 the wire running through the dense and fibrous ligamentum flavum.

The 7 French dilator is then placed over the guide wire and the wire is used to direct the dilator as it is gently but firmly pushed through the fascia,
20 paraspinous muscle, and ligamentum flavum, following the track of the wire toward the subarachnoid space. Advancement of the 7 French dilator is stopped and the dilator removed from the wire as soon as a loss of resistance is detected after passing the ligamentum
25 flavum. This is done in order to avoid advancing and manipulating this relatively rigid dilator within the subarachnoid space to any significant degree.

After the wire track is "overdilated" by the 7 French dilator, the 6 French dilator and cannula
30 sheath are assembled and placed over the guide wire. The 6 French dilator and cannula are advanced carefully into the subarachnoid space until the opening tip of

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the cannula is positioned 7 cm within the space. As with the 7 French dilator, the assembled 6 French dilator and cannula are directed by the wire within the lumen of the dilator. Position within the subarachnoid space is determined by premeasuring the device and is grossly confirmed by fluoroscopy. Great care is taken with manipulation of the dilators and cannula within the subarachnoid space to avoid misdirection and possible neurologic injury.

When appropriate positioning of the cannula is assured, the guide wire and the 6 French dilator are gently removed from the lumen of the cannula in sequence. Depending on the patient's position on the operating table, CSF flow through the cannula at this point should be noticeable and may be very brisk, requiring capping the cannula or very prompt placement of the capsule implant in order to prevent excessive CSF.

The encapsulated (transformed cells) is provided in a sterile, double envelope container, bathed in transport medium, and fully assembled including a tubular silicone tether. Prior to implantation through the cannula and into the subarachnoid space, the capsule is transferred to the insertion kit tray where it is positioned in a location that allowed the capsule to be maintained in transport medium while it is grossly examined for damage or major defects, and while the silicone tether is trimmed, adjusting its length to the pusher and removing the hemaclip™ that plugs its external end.

The tether portion of the capsule is mounted onto the stainless steel pusher by inserting the small

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diameter wire portion of the pusher as the membrane portion of the device is carefully introduced into the cannula. The capsule is advanced until the tip of the membrane reaches a point that is 2-10 mm within the cranial tip of the cannula in the subarachnoid space. This placement is achieved by premeasuring the cannula and the capsule-tether-pusher assembly, and it assures that the membrane portion of the capsule is protected by the cannula for the entire time that it is being advanced into position.

After the capsule is positioned within the cannula, the pusher is used to hold the capsule in position (without advancing or withdrawing) in the subarachnoid space while the cannula is completely withdrawn from over the capsule and pusher. The pusher is then removed from the capsule by sliding its wire portion out of the silicone tether. Using this method the final placement of the capsule is such that the 5 cm long membrane portion of the device lay entirely within the CSF containing subarachnoid space ventral to the cauda equina. It is anchored at its caudal end by a roughly 1-2 cm length of silicone tether that runs within the subarachnoid space before the tether exits through the dura and ligamentum flavum. The tether continues externally from this level through the paraspinous muscle and emerges from the lumbodorsal fascia leaving generally 10-12 cm of free tether material that is available for securing the device.

CSF leakage is minimized by injecting fibrin glue (Tissel®) into the track occupied by the tether in the paraspinous muscle, and by firmly closing the superficial fascial opening of the track with a purse-

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string suture. The free end of the tether is then anchored with non-absorbable suture and completely covered with a 2 layer closure of the skin and subcutaneous tissue.

- 5 The patient is then transferred to the neurosurgical recovery area and kept at strict bed rest, recumbent, for 24 hours postoperatively. Antibiotic prophylaxis is also continued for 24 hours following the implantation procedure.

10 Sequences

 The following is a summary of the sequences set forth in the Sequence Listing:

- SEQ ID NO:1 -- DNA sequence of oligo oCNTF-003
SEQ ID NO:2 -- DNA sequence of oligo oIgSP-018
15 SEQ ID NO:3 -- DNA sequence of IgSP-hPOMC fusion
SEQ ID NO:4 -- DNA sequence of IgSP-hPOMC- Δ ACTH fusion
SEQ ID NO:5 -- DNA sequence of oligo orTH-052
SEQ ID NO:6 -- DNA sequence of oligo orTH-053
SEQ ID NO:7 -- DNA sequence of oligo orTH-054
20 SEQ ID NO:8 -- DNA sequence of oligo orTH-078
SEQ ID NO:9 -- DNA sequence of oligo oIRES-057
SEQ ID NO:10 -- DNA sequence of oligo obDBH-065
SEQ ID NO:11 -- DNA sequence of oligo oIRES-bDBH-064
SEQ ID NO:12 -- DNA sequence of oligo oIRES-bDBH-066
25 SEQ ID NO:13 -- DNA sequence of oligo oIRE-068
SEQ ID NO:14 -- DNA sequence of oligo orTH Δ -073
SEQ ID NO:15 -- DNA sequence of oligo ohPOMC-IRES-069
SEQ ID NO:16 -- DNA sequence of rTH Δ 1-155
SEQ ID NO:17 -- DNA sequence of rTH Δ +KS
30 SEQ ID NO:18 -- DNA sequence of rTH Δ -IRES-bDBH
SEQ ID NO:19 -- DNA sequence of rTH Δ KS-IRES-bDBH

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- SEQ ID NO:20 -- DNA sequence of oligo ohPOMC-IRES-070
 SEQ ID NO:21 -- DNA sequence of oligo oIRES-rTHA-071
 SEQ ID NO:22 -- DNA sequence of oligo orIRES-rTHA-072
 SEQ ID NO:23 -- DNA sequence of IgSP-hPOMCACTH-IRES-
 5 rTHA-IRES-bDBH-068 fusion
 SEQ ID NO:24 -- DNA sequence oIRES-074
 SEQ ID NO:25 -- DNA sequence of oligo oZeocin-077
 SEQ ID NO:26 -- DNA sequence of oligo oIRES-Zeocin-075
 SEQ ID NO:27 -- DNA sequence of oligo oIRES-Zeocin-076
 10 SEQ ID NO:28 -- DNA sequence IgSP-hPOMCACTH-IRES-rTHA
 -IRES-bDBH-IRES-Zeocin-073
 SEQ ID NO:29 -- DNA sequence of proA+KS
 SEQ ID NO:30 -- DNA sequence of IRES fragment

Deposits

- 15 RINa/ProA/POMC/TH-IRES-DBH cells, transformed
 to produce a catecholamine, an enkephalin and an
 endorphin, as described above in the example (and in
 Table 2), named RINa/ProA/P030/P088, have been
 deposited. The deposit was made in accordance with the
 20 Budapest Treaty and was deposited at the American Type
 Culture Collection, Rockville, Maryland, U.S.A. on June
 7, 1995. The deposit received accession number
 CRL 11921.

- The foregoing description has been for the
 25 purpose of illustration and description only. This
 description is not intended to limit the invention to
 the precise form exemplified. It is intended that the
 scope of the invention be defined by the claims
 appended hereto.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: CytoTherapeutics, Inc. (For purposes of all
designated states except US)
Shou Wong (For purposes of US only)
Joel Saydoff (For purposes of US only)
- 10 (ii) TITLE OF INVENTION: PAIN CELL LINE
- (iii) NUMBER OF SEQUENCES: 30
- 15 (iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 1251 Ave. of the Americas
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20 (D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 10020-1104
- (v) COMPUTER READABLE FORM:
25 (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- 30 (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
35 (A) APPLICATION NUMBER: US 08/481,917
(B) FILING DATE: 07-JUNE-1995
- (viii) ATTORNEY/AGENT INFORMATION:
40 (A) NAME: Elrifi, Ivor R
(B) REGISTRATION NUMBER: 39,529
(C) REFERENCE/DOCKET NUMBER: CTT-29 CIP PCT
- (ix) TELECOMMUNICATION INFORMATION:
45 (A) TELEPHONE: 212 596-9000
(B) TELEFAX: 212 596-9090

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(2) INFORMATION FOR SEQ ID NO:1:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

15 (iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: cONTF-003

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

COOCCATCCG CGTCACCCCT AGAGTGGC TGT

33

25

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
30 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40

(vii) IMMEDIATE SOURCE:
(B) CLONE: oIgSP-018

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTCCCGGGA AAGCGAATT CAC

23

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(2) INFORMATION FOR SEQ ID NO:3:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 849 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 10 (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 15 (vii) IMMEDIATE SOURCE:
(B) CLONE: IgSP-hPMC
- 20 (ix) FEATURE:
(A) NAME/KEY: 5'UTR
(B) LOCATION: 1..43
- (ix) FEATURE:
25 (A) NAME/KEY: exon
(B) LOCATION: 44..89
- (ix) FEATURE:
30 (A) NAME/KEY: intron
(B) LOCATION: 90..168
- (ix) FEATURE:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 807..849
- 35 (ix) FEATURE:
(A) NAME/KEY: misc feature
(B) LOCATION: 43..186
(D) OTHER INFORMATION: /product= "IgSp region"
- 40 (ix) FEATURE:
(A) NAME/KEY: misc feature
(B) LOCATION: 187..806
(D) OTHER INFORMATION: /product= "hPMC region"
- 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GGATCGGGT CACCCCTAGA GTGAGCTGT GAGGGTCTT ACAATGAAT GCAGCTGGGT 60
 TATCTCTTC CIGATGGCAG TGGTTACAGG TAAGGGGCTC CCAAGTCCCA AACTTGAGGG 120
 5 TCCATTAACCT CTGTGACAGT GGCATCACT TTGCTTTCT TTCTACAGGG GTGATTGG 180
 CTTTCCGGG AAATGGGAC GAGAGGCTC TGACCGAGA CCCCAGGAG TAGTCAATG 240
 10 GCGACTTGG CTGGACCGA TTGGGCGGC GCAACAGCAG CAGCAGGGC AGCAGCGGG 300
 CAGGGCAGA GCGGAGGAC GTCTAGGG GCGAGACTG CGGGGCGTG CCTGAGGGG 360
 GCGCGAGCC CCGCAGGAT GGTGCGAGC CGGGGCGG CGAGGCGAG CGCTCTACT 420
 15 CCATGAGCA CTTGCGCTG GCGAGCGG TGGGAGAA GCGGCGCCA GTGAGGTGT 480
 ACGCTAAGG CGCGAGGAC GAGTGGGAG AGGCTTTCC CCTGAGTTC AAGAGGGAC 540
 20 TCACTGGCA GCGACTGGG GAGGAGATG GCGCGAGG CCTGCGAT GACGGCGAG 600
 GGGCGAGC CGACTGGAG CACAGCTGC TGGTGGGC CGAGAGAG GCGAGGGC 660
 CCTACAGGAT GGAGCTTTC CGTGGGCA GCGCGCCA GGACAGGC TAGCGGTT 720
 25 TCATGACTC CGAGAGAG CAGAGGCG TGGTAGCT GTTCAAAAC GGCATATCA 780
 AGAGCGCTA CAGAGGGC GAGTAGGC ACAGGGGC CCAGGGCTAC CCTCCCCAG 840
 30 GAGGTGAC 849

(2) INFORMATION FOR SEQ ID NO:4:

- 35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 525 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 40 (ii) MOLECULE TYPE: DNA (genomic)
 (iii) HYPOTHETICAL: NO
 (iv) ANTI-SENSE: NO
 45 (vi) IMMEDIATE SOURCE:

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(B) CLONE: IgSP-hPOMC/ACTH

(ix) FEATURE:

(A) NAME/KEY: 5'UTR

5 (B) LOCATION: 1..43

(ix) FEATURE:

(A) NAME/KEY: exon

10 (B) LOCATION: 44..89

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 90..168

(ix) FEATURE:

(A) NAME/KEY: exon

15 (B) LOCATION: 169..482

(ix) FEATURE:

(A) NAME/KEY: 3'UTR

20 (B) LOCATION: 483..525

(ix) FEATURE:

(A) NAME/KEY: misc feature

25 (B) LOCATION: 44..188

(D) OTHER INFORMATION: /product= "IgSP region"

(ix) FEATURE:

(A) NAME/KEY: misc feature

30 (B) LOCATION: 189..482

(D) OTHER INFORMATION: /product= "hPOMC region"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

35 GGATCGGGT CACCCCTAGA GTGAGCTGT GACGGTCTT ACAATGAAT GCAGCTGGT 60
 TATCTCTTC CIGATGGAG TGGTTACAG TAAGGGCTC CCAAGTCCA AACTTGAGG 120
 40 TCATAAACT CTGIGACAGT GGCATCACT TTGCCTTCT TTCTACAGG GIGATTGG 180
 CTTTCCGGC CTTCCCCCTG GAGTTCAAG GGGAGCTGAC TGGCCAGGA CTCCGGAGG 240
 GAGATGGGC CCAAGGCTT GCGATGAG GCGAGGGC CAGGCGGAC CTGAGGACA 300
 45 GCTGCTGGT GCGGCGGAG AAGAGGAG AGGCGGCTA CAGGATGG CACTTCCCT 360

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GGGGCAGCCC GCGCAGGAC AAGCGCTAG GGGTTTCAT GACCTCGAG AAGAGCCAGA 420
 CGCCCCCTGGT GAGCGTGTC AAAAAGGCA TCATCAAGAA CGCTACAAG AAGGGCGAGT 480
 5 GAGGGCACAG CGGGCCCCAG GGCTACCTC CCCCAGGAGG TGAC 525

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 10 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
 15 (iii) HYPOTHETICAL: NO
 (iv) ANTI-SENSE: NO
 20 (vii) IMMEDIATE SOURCE:
 (B) CLONE: orTH-052
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
 25

CCCAAGCTTG CACTATGCC ACCCCCAGG 30

30 (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 35 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 40 (iv) ANTI-SENSE: NO
- (vii) IMMEDIATE SOURCE:
 45 (B) CLONE: orTH-053

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCGATCCT ATGCATTAG CTAATGGCAC

30

5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

10

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

20

(vii) IMMEDIATE SOURCE:

(B) CLONE: orTH-054

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCCAGCTTA TGGTCCCCG GTTCCGAGA

30

30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

35

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

40

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

45

(B) CLONE: orTH-078

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCCAGCTTC GGCACCATGG TCCCCTGGTT CCC

33

5 (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

15 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

20 (vii) IMMEDIATE SOURCE:
(B) CLONE: oIRES-057

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

25

AAAGGATCG CCCCCTCCTCC TCCCCCCCC

30

(2) INFORMATION FOR SEQ ID NO:10:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

40 (iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: obEH-065

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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AAAGGGGGG CCCACGTCA GCGTTTGCC

30

(2) INFORMATION FOR SEQ ID NO:11:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

10

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

15

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

20

(B) CLONE: oIRES-bDEH-064

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

25 CTGGCCACA CCAATGACGG CACGGGGTG

30

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

30

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40

(vii) IMMEDIATE SOURCE:

(B) CLONE: oIRES-bDEH-066

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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CGGGTGGG TACATGGTIG TGCAAGCTT

30

(2) INFORMATION FOR SEQ ID NO:13:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

15 (iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: oIgSP-068

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AAAGATATCG CGGCGGGTIC ACCCTAGAG

30

25

(2) INFORMATION FOR SEQ ID NO:14:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40

(vii) IMMEDIATE SOURCE:
(B) CLONE: orTHD-073

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATACACCTGG TCAGAGAGC CCGGG

25

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(2) INFORMATION FOR SEQ ID NO:15:

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 10 (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 15 (vii) IMMEDIATE SOURCE:
 (B) CLONE: chPMC-IRES-069
- 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGGGGGAGGG AGAGGGGGCC GCTGTGGGCT

30

- 25 (2) INFORMATION FOR SEQ ID NO:16:
- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1030 base pairs
 (B) TYPE: nucleic acid
 30 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- 35 (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 40 (vii) IMMEDIATE SOURCE:
 (B) CLONE: rTHD
- (ix) FEATURE:
 (A) NAME/KEY: 5'UTR
 45 (B) LOCATION: 1..6
- (ix) FEATURE:

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(A) NAME/KEY: exon
(B) LOCATION: 7..1017

(ix) FEATURE:

5 (A) NAME/KEY: 3'UTR
(B) LOCATION: 1018..1030

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

10	AAGCTTATGG TCCCTGGTT CCCAGAAAA GTGTGGGAT TGGACAGTG TCACCACTG	60
	GTACCAAGT TTGAACCTGA TCTGGACCTG GACCAACGG GCTTCTCTGA CCAGGIGTAT	120
15	CGCCAGGTC GGAAGCIGAT TGCAGAGATT GCTTCCAGT ACAAGCAAGG TGAACCAAT	180
	CCCCATGTTG AATAACACG GGAAGAGATT GCTACCTGGA AGGAGGATA TGTCAGCTG	240
	AAGGGGCTCT ATGCTACCA TGGCTGGGG GAGCACTGG AGGGTTTCCA GCTTCTGGAA	300
20	CGGTACTGTG GCTACCGAGA GGACAGCATC CCACAGCTGG AGGACGIGTC CGGCTCTCTG	360
	AAGGAGGGA CTGGCTTCCA GCTGGGACC GTGGCCGCTC TACTGTGGC CGGTGATTTT	420
25	CTGGCAGTC TGGCTTCCG CGGTGTTCAA TGCACCAAGT ATATCGGCA TGCTCTCTCA	480
	CGTATGCATT CACCTGAGC GGACTGCTC CATGAGCTG TGGACATGT AACCATGTTG	540
	GCTGACCGCA CATTGGCCA GTTCTCCAG GACATGGAC TTGCATCTCT GGGGCGCTCA	600
30	GATGAGAAA TTGAAACT CTCCAGGCTG TACTGGTCA CTGTGGAAAT CGGGCTATGT	660
	AACAGAAATG GGGAGCTGA GCTTATGGT GCAGGCTGC TGCTTCTCA CGGAGGCTC	720
35	CTGCACTCC TGTCAGGA GCTGAGGTC CGAGCCTTG AACCAGAC ACCAGCTGIG	780
	CAGGCTACC AAGTCAAC CTACAGCT GTGTACTTG TGTCGAGAG CTTCATGAC	840
	GCCAGGACA AGCTCAGGA CTATGCTCT GGTATCCAG GGCATTCTC TGTAAGTTT	900
40	GACCGTACA CACTGGCAT TGAGTACTG GACAGGCTC ACACCATCA GGGCTCTTG	960
	GAGGGGTCC AGGATGAGT GCACACCTG GGCAGGCAC TGAGTGCAT TAGCTAAATG	1020
45	CATAGGATCC	1030

(2) INFORMATION FOR SEQ ID NO:17:

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(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1037 base pairs
 (B) TYPE: nucleic acid
 5 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

10 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15 (vii) IMMEDIATE SOURCE:
 (B) CLONE: rTHDS

(ix) FEATURE:
 (A) NAME/KEY: 5'UTR
 20 (B) LOCATION: 1..13

(ix) FEATURE:
 (A) NAME/KEY: exon
 (B) LOCATION: 14..1024

25 (ix) FEATURE:
 (A) NAME/KEY: 3'UTR
 (B) LOCATION: 1025..1037

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

APGCTTCGCC ACCATGGTCC CCTGGTTCAC AAGAAAGTGG TGGGAATTGG ACAAGIGTCA	60
35 CCACCTGGTC ACCAGTTTG AACCIGATCT GGACCTGGAC CACCCGGGCT TCTCTGACCA	120
GGIGTATCGC CAGGTGGGA AGCTGATTGC AGAGATTGCC TTCCAGTACA AGCAGGGTGA	180
ACCAATTCCC CATGTGGTAT ACACAGCGGA AGAGATTGCT AACTGGGAGG AGGTATATGT	240
40 CACGCTGAAG GGCTCTATG CTAACCATGC CTGGGGGAG CACCTGGAGG GTTTCAGCT	300
TCTGGAACGG TACTGTGGCT AAGGAGAGGA CAGCATGCCA CAGCTGGAGG AAGTGTCCCG	360
45 CTCTCTGAAG GAGGGACTG GCTTCAGCT GCGACCGGIG GCGGCTCTAC TGTCCGCGCG	420
TGATTTCTCG GGCAGTCTGG CCTTCGGGT GTTCAATGC AACAGTATA TCGGCATGC	480

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CTCTCTACCT ATGCATTAC CTGAGGCGA CTGCTGOCAT GAGCTGTGG GACATGTACC 540
 CATGTTGGCT GACCGCAT TGGCCAGTT CTTCCAGGAC ATTGGACTTG CATCTCTGGG 600
 5 GGGCTCAGAT GAAGAATTG AAAAATCTCT CAGGTGTAC TGGTTCACIG TGAATTCGG 660
 GCTATGTAAA CAGAATGGG AGCTGAGGC TTATGGTGA GGGCTGCTGT CTTCCTAGG 720
 10 AGAGCTCTG CACTCCCTGT CAGAGGAGC TGAGGTGGA GCTTTTGACC CAGACACAGC 780
 AGCTGTGAG CCTACCAAG ATCAAACTA CCAGCTGTG TACTTTGIGT CCGAGAGCTT 840
 CAATGAGGC AAGGACAGC TCAGGACTA TGGCTCTGT ATCCAGGOC CATCTCTGT 900
 15 GAAGTTTAC CGGTACAC TGGCATTGA CGTACGGAC AGGCTCACA CCATCCAGG 960
 CTCTTGGAG GGGTCCAGG ATGAGCTGA CAGCTGGOC CAGGACTGA GGGCATTAG 1020
 20 CTAAATGCAT AGGATOC 1037

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 25 (A) LENGTH: 3425 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 30 (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 35 (vii) IMMEDIATE SOURCE:
 (B) CLONE: rTH-IRES-bDEH
- 40 (ix) FEATURE:
 (A) NAME/KEY: 5'UTR
 (B) LOCATION: 1..6
- 45 (ix) FEATURE:
 (A) NAME/KEY: exon
 (B) LOCATION: 7..1017

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(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1018..1617

5 (ix) FEATURE:
 (A) NAME/KEY: exon
 (B) LOCATION: 1618..3411

10 (ix) FEATURE:
 (A) NAME/KEY: 3'UTR
 (B) LOCATION: 3412..3425

(ix) FEATURE:
 (A) NAME/KEY: misc feature
 (B) LOCATION: 1025..1617
 (D) OTHER INFORMATION: /product= "IRES sequence"

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

20

AAGCTTATGG TCCCTGGT CCACAGAAA GTGTGGGAT TGGACAAGTG TCACCACTG	60
GTCAACAGT TTGACCTGA TCTGGACCTG GACCAACCG GCTTCCTGA CCAGGIGTAT	120
CGCCAGGTC GGAAGCTGAT TGCAGAGATT GCTTCCAGT ACAAGCAAGG TGAACCAATT	180
CCCATGTGG AATACACAGC GGAAGAGATT GCTACCTGGA AGGAGGTATA TGTCAGCTG	240
AAGGGCTCT ATGCTACCA TGCTGCGG GAGCACTGG AGGTTTCCA GCTTCGGAA	300
30 CGGTACTGIG GCTACCGAGA GGACAGATC CCACAGCTG AGGAGGTC CGCTTCTTG	360
AAGGAGCGA CTGGCTTCCA GCTGGAGCC GTGGCGGTC TACTGTCCG CGGTATTTT	420
35 CTGGCCAGTC TGCCCTTCCG CGGTATTCAA TGCAACAGT ATATCCGCA TGCTCTCA	480
CCATATGATT CACCTGAGC GACTGCTGC CATGAGCTGT TGGACATGT AACCATGTTG	540
GCTGACCGA CATTGCGCA GTCTCCAG GACATGGAC TTGCATCTCT GGGGCTCA	600
40 CATGAGAAA TTGAAAAC CTCCAGGTC TACTGGTCA CTGGGAATT CGGCTATGT	660
AAACAGATG GGGAGCTGAA GCTTATGT GCAGGCTGC TGCTTCTCA CGGAGGCTC	720
45 CTGCACTCC TGTCAGGGA GCTGAGGTC CGAGCTTTG AACCAGAC AGCAGCTGIG	780
CAGCCCTAC AGATCAAC CTACAGCT GGTACTTTG TGTCGAGG CTTCATGAC	840

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	GCCAGGACA AGCTCAGGA CTATGCTCT CGTATCCAGC GGCATCTC TGTAAGTTT	900
	GACCGTACA CACTGGCAT TGAGTACTG GACAGGCTC ACAACATCA GCGCTCTTG	960
5	GAGGGGTTC AGGATGAGCT GCACGCTG GCGACGAC TGAGTGCAT TAGCTAATG	1020
	CATAGGATC GCGCTCTC CTGCGGCTC CTGAGGTA CCGGCGAG CCGCTTGAA	1080
10	TAGGCGGT GCGGTTGT CTATAGTA TTTCACCA TATGCGTC TTTTGCAAT	1140
	GTCAGGCTC GCAACCTG CCGCTCTC TTGAGGCA TCTGAGGG TCTTTGCT	1200
	CTGCGGAG GATGCGAG TCTGTGAT GTGTGAGG AAGGCTTC TCTGAGCT	1260
15	TCTGAGAC AACACGTC TGAGGAGC CTGTCAGC AGCGGAC CCGCTGCG	1320
	GACAGGCTC TCTGCGCA AAGCGCT GTATAGTA CCGTCAAA GCGGCACA	1380
20	CGGAGGCT AGGTTGAG TTGGATAGT GCGGAGAG TCAATGCT CTCTCAGC	1440
	GTATCAACA AGGGCTGA GATGCGAG AAGGACCT ATTGTATGG ATCTGCTG	1500
	GCGCTCGT GCATGCTT TACATGCT TAGTCAGT TAAAAAGT CTAGGCTC	1560
25	CGACGAGG GAGGCTGT TCTTTTGA AACACGAT ATAGCTGC CACACCATG	1620
	TAGGCTCG CCGTGGCT CTCTGCTC ATCTGCTG CCGCTGCA GCGCTCGCT	1680
30	CGCGGAGA GCGCTTCC CTCTCCTC CCGTGGCT CCGAGGAG CCGGAGCTG	1740
	TCTGGAACA TCGCTATG CAGGAGAC ATCTCTTC AGCTCTGT GCGGAGCTC	1800
	AAGCTGGT TCTGTTGG GATGCGAC CAGGAGAG TGGAGATG TGCTTGGT	1860
35	GTCTCTGA CTGACGGA CCGGCTAC TTGGGGAT CCGGAGTA CAGAGGGG	1920
	CAGTCCAC TGACTCCA GAGGATAC CAGCTCTC GGCACAGG GACTCCAA	1980
40	GCGCTGAC TCTCTTCA GCGCTTTT GCGCTGTG ACGGAGCA CTCTCTC	2040
	GAGGCGCA CCGTCCCT GGCTATGA TCTGAGG AGCGCTCG GTGCTGGG	2100
	TCTCAACA CATCGCTT GCACGCGG CTGACAGG TGAGCTCT GAGCGGAG	2160
45	ATCGGAGC CCGCTCTC CCGGAGAG CCGCATG AGTCCGCG CCGGAGCT	2220

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CTCAT0000G G0CAGCAGAC CAGCTACTGG TGCTA0G1GA 00GAGCT00C G5A0G3CTTC 2280
 0000G3CACC ACAT0G1CAT GTAGAG00C AT0GTCA00G AGG3CA00GA G0G0CTGGTG 2340
 5 CAC0CATGG AGGTCTT0CA GTG0G00G0C GAGT0GAGA 0CAT0000CA CTTCAG0GGG 2400
 00CTG0GACT 0CAAGATGA 000GAG0GG CTCACTTCT 00GTCA0GT GCTG00G0C 2460
 TGG000CTGG G0G0CAGGC CTTTACTAC 0CAGAGGAG CAGG0CTGGC CTTCG3GGG 2520
 10 00G0CT0CT 0CAGATTCT 00G0CTGGA GTTACTTAC ACA00CACT GGATATAA 2580
 G00G0G0G ACT0CT0GG CAT0G0CTG TACTACAGG CTG0GCTGG G0GCTT0GAC 2640
 15 G0G0CATCA TGGAGCTGG 0CTG0GTAC A0G000G1GA TGG0CAT00C 00G0AGGAG 2700
 A0G00CTTG T0CTCA00G CTACTG0AG GACAAGTGA 00AGCTGGC 0CTG00G0C 2760
 TCAGGATTC ACATCTT0G CTCTAGCTC CACA0G0C TGAC0G00G GAGGTGGTC 2820
 20 ACAGTCTGG 0CAGG0GG 00GGAGACA GAGAT0G1GA ACAGGACAA 0CACTACAGC 2880
 0CACACTTC AGGAGAT0G CATGTGAAG AAGGT0GTGT CTGT0CAGC GGGAG0GTG 2940
 25 CTCATCACT CTTCACATA CACAGGGA GACAGGAGC TGG0CAT0GT GGGG0CTTC 3000
 GGGAT0CTGG AGGAGATGT GGTCACTAT GTGCACTACT A0000CAGC GCAGCTGGAG 3060
 CTCIG0AGA G0G00G1GA 00CTG0CTTC CTGCAAGT ACTT0G0CT 0GTGACAGG 3120
 30 TTCAACAGG AGGAGTCTG CACCTG00C CAGG0TCTG T00CTGACA GTTGT0CTC 3180
 GTG00CTGA ACT0CTTAA 00G0AGGTG CTCAGG00C TGTAGGCTT 0GCACCATC 3240
 35 T0CATGACT GCAACAGGT CTCG00GTC 0GCTT0CAGG G0GAGTGA T0G0CAG0C 3300
 CTG0CTGAGA T0GTGT0CAG GTTGAAGAG 00CA000CT ACTG00CAGC CAG0AGGCT 3360
 CAGAG000G 00G0000AC 0GTGCTGAC ATCAGTGGG GCAAGGCTG AAGTGG0G 3420
 40 G0GC 3425

(2) INFORMATION FOR SEQ ID NO:19:

- 45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 3432 base pairs
 (B) TYPE: nucleic acid

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(C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

5 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10 (vii) IMMEDIATE SOURCE:
 (B) CLONE: pTHIKS-IRES-bDEH

(ix) FEATURE:
 15 (A) NAME/KEY: 5'UTR
 (B) LOCATION: 1..13

(ix) FEATURE:
 20 (A) NAME/KEY: exon
 (B) LOCATION: 14..1024

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1025..1624

25 (ix) FEATURE:
 (A) NAME/KEY: exon
 (B) LOCATION: 1625..3418

30 (ix) FEATURE:
 (A) NAME/KEY: 3'UTR
 (B) LOCATION: 3419..3432

(ix) FEATURE:
 35 (A) NAME/KEY: misc feature
 (B) LOCATION: 1032..1624
 (D) OTHER INFORMATION: /product= "IRES sequence"

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAGCTTGGCC ACCATGGTCC CCTGGTTCAC AAGAAAGTGG TGGGAATTGG ACAAGTGTCA	60
CCACCTGGTC ACCAAGTTTG ACCCGATCTT GGACCTGGAC CACCCGGGCT TCTCTGACCA	120
GGTGATATGC CAGGTGGGA AGCTGATTGC AGAGATTGCC TTCCAGTACA AGCAAGGTGA	180

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	ACCAATTOOC CATGIGGAAT ACACAGOGGA AGAGATTGCT AACTGGAAGG AGGTATATGT	240
	CACCGTAGAG GGCCTCTATG CTACCCATGC CTGCGGGGAG CACTGGGAGG GTTTCAGCT	300
5	TCTGGAAGG TACTGTGGCT AOCGAGAGGA CAGCATOCCA CAGCTGGAGG ACGTGTCCCG	360
	CTTCTTGAAG GAGGGACTG GCTTCAGCT GCGACCGTG GCGGCTCTAC TGTCCGCGCG	420
	TGATTTCTIG GCGAGTCTGG CCTTCGCGGT GTTTCATGC AOCAGTATA TCCGCCATGC	480
10	CTCCTCACT ATGCATTAC CTGAGCGGA CTGCTGCAT GAGCTGTGG GACATGTACC	540
	CATGTGGCT GACCGACAT TGGCCAGTT CTCACGGAC ATGGACTTG CATCTCTGG	600
15	GGCTCAGAT GAAGAAATG AAAACCTC CACGGTGTAC TGGTTCCTG TGAATTCGG	660
	GCTATGTAA CAGATGGG AGCTGAGGC TTATGGTGA GCGCTGCTGT CTTCCTAGG	720
	AGAGCTCTG CACTCCCTGT CAGAGGAGC TGAGGTCCA GCGTTTGAC CAGACACAG	780
20	AGCTGTGAG CCTACCTAG ATCAAACTA CCAGCTGTG TACTTTGTG CCGAGAGCTT	840
	CAATGAGGC AAGGACAGC TCAGGACTA TGCTCTGT ATCAGCGCC CATCTCTGT	900
25	GAAGTTTGC CCGTACACAC TGGCATTGA CGTACTGGAC AGCCCTACA CCATCCAGG	960
	CTCCTGGAG GGGTCCAGG ATGAGCTGA CACCTGGCC CAGGACTGA GTCGATTAG	1020
	CTAAATGCAT AGGATCCGC CCTCTCCCTC CCCCCCCT AAGTTACTG GCGAAGCG	1080
30	CTTGGATTA GCGGGTGTG CGTTGTCTA TATGTATTT TCCACATAT TCGGTCTTT	1140
	TGGCAATGT AGGGCCCGA AACTGGCC TGCTCTCTG ACGAGCATC CTAGGGTCT	1200
35	TTCCCTCTC GCGAAGGAA TCGAGGCT GTTGAATGC GTGAGGAG CAGTCCCT	1260
	GGAGCTTCT TGAAGACAA CAGCTCTGT AGCGACCTT TCGAGGAGC GGAACCC	1320
	AACTGGGAC AGGTCCCTCT GCGGCCAAA GCGAGGTA TAGATACAC CTGCAAGGC	1380
40	GCGACATCC CAGTCCAGG TTGTGAGTG GATAGTTTG GAAAGATCA AATGGCTCT	1440
	CTCAGGTA TTCAACAGG GCGTAGGA TCGCAGAG GTACCCATT GTATGGGATC	1500
45	TGATCTGGG CCTCGGTGA CATGCTTAC ATGTTTATG TCGAGGTAA AAAAGTCTA	1560
	GGCCCCCGA ACGAGGGA CGTGGTTTC CTTGAAAA CAGATGATA AGCTTGCAC	1620

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AACCATGTAC GGCACCGGG TGGGCGCTT CCTGGTCATC CTGGGGCTG CACTGCAGG 1680
 CTGGGCTOOC GGGGAGAGC CCTTGGCTT CCATATCCC CTGGACCGG AGGGGAGCT 1740
 5 GGAGCTGTCC TGGACATCA GCTATGGCA GGAGACATC TACTTCCAGC TCTGGTGG 1800
 GGAGCTCAG GCTGGGTCC TGTGGGGAT GTGGACCA GGGGAGCTG AGATGCTCA 1860
 10 CTGGGGTGG CTCTGGCTG ACAGGAGG CGCTACTTT GGGGATGCT GGAGTACA 1920
 GAGGGGAG GTCCACTGG ACTCCAGCA GGATTACAG CTCTGGGG CACAGAGG 1980
 TCCAGAGGC CTGTACCTG TCTTCAGAG GCTTTTGGC AACTGGAC CCACGACTA 2040
 15 CCTCATGAG GAGGCGAG TCCACTGGT GTATGGATC CTGGAGAGC CGCTGGGT 2100
 GCTGGAGTC ATCAACATC CCGGCTTGA CAGGGGCTG CAGAGGCTG AGCTGCTGA 2160
 20 GGCAGCATC CCGAGGGG CCTGGGGC GGCAGGGC ACCATGGAG TCGGGGCC 2220
 CGAGTCTC ATCCCGGC AGCAGACAC GTCTGGGT TACGTAGG AGCTCCGGA 2280
 CGGCTTCCC CGGACACA TGTCACTGA CAGGCGATC GTACCGAG GCAAGAGGC 2340
 25 GCTGGTCC CACATGGAG TCTTCAGTG CGCGGGAG TTGAGACA TCCCGACT 2400
 CAGGGGCC TCGACTCA AGATGAGC GAGGGCTC AACTCTGC GTACGTGT 2460
 30 GCGGGCTGG GCGTGGGG CCGAGGCTT TTCTACCA GAGGAGCAG GCTGGCTT 2520
 CGGGGGGCC GGTCTCTCA GATTCTCG CCTGGAGTT CACTACACA ACGACTGGT 2580
 GATACAGGC CGCGGACT CCTGGGCT CCGCTGTAC TACAGGCTG CGCTGGGG 2640
 35 CTTCAGCG GGCATCAG AGCTGGCT GCGTACAG CCGGTGATG CCATCCCC 2700
 GCAGGAGG GCTTGTCT TACGGCTA CTGACGGC AAGTCCAC AGCTGGCT 2760
 40 GCGGGCTCA GGGATACA TCTGGCTC TACCTCAC AGCAGCTGA CCGGGGGA 2820
 GGCTGACA GTCTGGCA GGGAGGGG GGCACAGAG ATGTGACA GGCACACA 2880
 CTACAGCA CACTTCCAG AGATCCAT GTGAGAGG GTGGGTCTG TCCAGGGG 2940
 45 AGAGTCTC ATCACTCT GCATACAA CAGGAGG AGAGGCTG CCGGTGG 3000

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GGGCTTGGG ATCTGGAGG AGATGTGGT CAACTATGTG CACTACTAC CCCAGAGCA 3060
 GCTGGAGCTC TGCAAGAGG CCGTGGACC TGGCTTCTG CACAAGTACT TCGGCTGTG 3120
 5 GAACAGGTC AACAGGAGG AAGTCTGCAC CTGCCCCAG GGTCTGTGC CTGAGCAGT 3180
 TGGCTTGGT CCGTGGACT CCTTCAACG CGAGGTGCTC AAGGCTGTG ACGGCTTGC 3240
 ACCATCTTC ATGCACTGA ACAGTCTC GGGGTTCG TCCAGGGG AGTGGATG 3300
 10 GCAGGCTG CCGTGAATG TGTGAGGT GGAAGAGC ACCCTCACT GGCAGCAG 3360
 CCAGGCTAG AGCCCCGG GGGCAGGT GCTGACATC AGTGGGGCA AAGGCTGAC 3420
 15 GTGGGGGGC GC 3432

(2) INFORMATION FOR SEQ ID NO:20:

20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

30

(vii) IMMEDIATE SOURCE:

(B) CLONE: chPMC-IRES-070

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGGGCAGC GGGGCTCT CCTTCCCCC

30

40 (2) INFORMATION FOR SEQ ID NO:21:

45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

5 (iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: oIRES-rTHD-071

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GAACCAAGGG ACCATGGTGTG TGGCAAGCTT

30

15

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

20

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

30

(vii) IMMEDIATE SOURCE:

(B) CLONE: oIRES-rTHD-072

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CTTGCCACAA CCATGGTCC CTGGTCCCA

30

40

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4499 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

45

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- 5
- (vii) IMMEDIATE SOURCE:
(B) CLONE: pmtc-th-dbh fusion
- 10 (ix) FEATURE:
(A) NAME/KEY: 5'UTR
(B) LOCATION: 1..43
- (ix) FEATURE:
15 (A) NAME/KEY: exon
(B) LOCATION: 44..89
- (ix) FEATURE:
20 (A) NAME/KEY: intron
(B) LOCATION: 90..168
- (ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 169..482
- 25 (ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 483..1080
- 30 (ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1081..2091
- (ix) FEATURE:
35 (A) NAME/KEY: intron
(B) LOCATION: 2092..2691
- (ix) FEATURE:
(A) NAME/KEY: exon
40 (B) LOCATION: 2692..4485
- (ix) FEATURE:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 4486..4499
- 45
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

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	GCGGCGCGT CAGCCCTAGA GTGAGCGTGT GAGGGTCCIT ACAATGAAAT GCAGCTGGGT	60
	TATCTTCITC CIGATGGCAG TGGTTACAGG TAAGGGGCTC CCAAGTCCCA AACTTGAGGG	120
5	TCCATAAAGT CIGTGACAGT GGCATCACT TTGCTTTCT TTCTACAGGG GIGAAITGG	180
	CTTTCGGGC CTTCCCGCTG GAGTTCAGA GGGAGCTGAC TGGCCAGCGA CTCCGGGAGG	240
10	GAGATGGGCG CGAGCGGCGT GCGATGAGG GCGCAGGGGC CCAGCGCGAC CTGGAGCACA	300
	GCTTCCTGGT GCGCGCGAG AAGAAGGAG AGGGGCGCTA CAGGATGGAG CACTTCGGCT	360
	GGGGCAGGCG GCGCAGGAC AAGCGCTAG GCGGTTTCAT GAGCTCGAG AAGAGCGAGA	420
15	CGCGGCTGGT GAGGCTGTC AAAAGCGCA TCATCAGAA CGCTACAGG AAGGCGAGT	480
	GAGGGCAGAG CGGGGCGCTC TCCCTCGCG CCGCTAAG TTACTGGCG AAGCGCTTG	540
20	GAATAGGCG GGTGTGGTT TGCTATATG TTATTTTCCA CCATATGGC GTCTTTTGGC	600
	AATGTAGGG CCGGAAAC TGGCGCTGTC TTCTGAGA GCATTCCTAG GGGCTTTTC	660
	CCCTCGGCA AAGGAATGA AGGTCTGTG AATGTGGGA AGGAGCGAGT TCCCTGGAA	720
25	GCTCTTGAA GACAACAC GTCTGTAGG ACGCTTTGA GCGAGGGAA CCGCGAGCT	780
	GGGACAGGT GCTCTGGG CCAAGCGCA CGGTATAG ATACCGCTC AAGGGGGCA	840
30	CAGCGGAGT GCGAGTGT GAGTGGATA GTTGTGAAA GAGTCAATG GCTCTCTCA	900
	AGGATATCA ACAAGGGCT GAGGATGCG CAGAGGTAC CCGATGTAT GGGATCTGAT	960
	CIGGGGCTC GGTCACATG CTTTACATGT GTTAGTGA GGTAAAAA CGCTAGGCG	1020
35	CCCGAAGCA CGGGAGGTG GTTTCTCTT GAAAAACAG ATGATAGCT TGGCACAAC	1080
	ATGGTCCCT GGTCCGAG AAAAGTGTG GATTTGACA AGGTGACCA CCGGTGAC	1140
40	AAGTTGACC CIGATCTGA CCGGACAC CCGGCTTCT CIGAGGAGT GTATCGGAG	1200
	CGTGGGAGC TGATGCGA GATTGCTC CAGTACAGC ACGGTGACC AATTCCCAT	1260
	GTTGATACA CAGCGAGA GATTGCTAC TGGAGGAGG TATATGAC GCTGAGGGC	1320
45	CTCTATGCTA CCGATGCTG CCGGAGAC CCGAGGGT TCCAGCTCT GGAAGGTAC	1380

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TGIGGCTACC GAGAGGACAG CATOOACAG CTGGAGGAG TGTOOOGCTT CTGAAGGAG 1440
 CGGACTGGCT TOCAGCTGOG AOOOGTGGOC GGTCIACITGT OOGOOOGTGA TTTICTGGOC 1500
 5 AGTCTGGSOCT TOOGOGTGT TCAATGACC CAGTATATCC GOCATGCTC CTCACCTATG 1560
 CATTCAOCTG AGOOGGACTG CTGOCATGAG CTGTGGGAC ATGTACOCAT GTTGGCTGAC 1620
 CGCACATTG CCGAGTCTC CCAGGACAT GACTTGCAT CTCIGGGGC CTCAGATGA 1680
 10 GAAATGAAA AACTCTOAC GGIGTACTGG TTCACIGGG AATTOGGCT ATGTAAACAG 1740
 AATGGGGAGC TGAAGGCTA TGGTGAGGG CTGCTGTCTT CCTAGGAGA GCTOCTGCAC 1800
 15 TOOCIGTCAG AGGAGCTGA GGTOOGAGOC TTGACOCAG ACACAGCAGC TGTCAGGOC 1860
 TACCAAGATC AAPCTACCA GOCIGTGTAC TTTGTGTOG AGAGCTTCAA TGAOGGCAAG 1920
 GACAGCTCA GGAATATGC CTCGTGTATC CAGOGOCAT TCTCTGTGA GTTGAOOG 1980
 20 TACACTGAG CCATGAGGT ACTGGACAGC OCTCACACA TOCAGGCTC CTGGAGGGG 2040
 GTCCAGGATG AGCTGCACAC COTGGOCAC GCCTGTGTG CCATTAGCTA AATGCATAGG 2100
 25 ATTOGGOOCT CTTOCTOOC OOOOCTAAC GTTACTGGOC GAAGOGCTT GGAATAAGC 2160
 OGGTGTGGT TTGTCTATAT GTTATTTTC ACCATATTGC CGTCTTTTG CAATGTGAGG 2220
 GOOOGAAC CTGGOOCTGT CTCTGTAGC AGCATTOCTA GGGTCTTTC CCTCTOGOC 2280
 30 AAAGGAATGC AAGGCTGT GTTGTGTG AGGAAGCAG TTCTCTGGA AGCTCTTGA 2340
 AGACAACAA CGTCTGTAGC GACCTTTGC AGGCAGGGA AOOOOOACC TGGGACAGG 2400
 35 TGCTCTGGG GOCAAAAGC AGGTGTATA GATACACTG CAAGGGGGC ACFAOOOCAG 2460
 TGCCAGTGT TGAGTGGAT AGTGTGGAA AGAGTCAAT GCTCTOCTC AAGGTATTC 2520
 APCAAGGGC TGAAGGATC CCAGAGGTA CCGATTGTA TGGGATCTGA TCTGGGSOCT 2580
 40 CGGTGCACAT GCTTACATG TGTGTAGTG AGGTAAAAA AGGTCTAGC CCGOOGAOC 2640
 ACGGGAGGT GGTTTTCTT TGAFAACAC GATGATAAGC TTGOCACAC CATGTACGGC 2700
 45 ACGGGGGG CCGTCTOCT GTTCATCTC GTGGCTGCAC TGCAGGGCTC GGTTOOGOC 2760
 GAGAGOOCT TOOCTTACA CATOOOCTG GAOOOGAGG GAOOCTGGA GCTGTCTGG 2820

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	AACATCAGCT ATGCGCAGGA GACCATCTAC TTCCAGCTCC TGGTGCGGGA GCTCAAGGCT	2880
	GGGTGCTGT TTGGGATGTC GGACCGAGGG GAGCTGGAGA ATGCTGACTT GGTTGGTCTC	2940
5	TGGACTGACA GGGACGGGCG CTACTTTGGG GATGCTTGA GTCACAGAA GGGGAGGTG	3000
	CACTGGACT CCCAGCAGGA TTACAGCTT CTGGGGGAC AGAGGACTCC AGAGGGCTG	3060
10	TACCTGCTCT TCAAGAGGC TTTTGGCACC TGTCACCCA AGACTAAGT CATGAGGAC	3120
	GGCAAGGTC AACTGGTGTA TGGATTCTG GAGGAGGCG TCGGTGCTT GGAGTCAATC	3180
	AACACATCG GCTTGCACAC GGGCTGCAG AGGGTGCAG TCGTGAGCC CAGCATCCCC	3240
15	AAGCGGGCC TGGGCGGA CAGCGCAC ATTGAGATC GGGGGGGA CGTCTCATC	3300
	CCGGGACG AGACAGGTA CTGGTCTAC GTGACGAGC TCCCGGAGG CTTCGGGG	3360
20	CACACATCG TCATGTAGA GCGATGTC ACGAGGGCA AGAGGGCTT GGTGACAC	3420
	ATGAGGCTT TCCAGTGGC CGCGAGTTC GAGACATCC CCACTTCAG CGGGGCTGC	3480
	GACTCCAGA TGAAGGCA GGGCTCAAC TTCTGGGTC AGTGTGTC CGCTGGGCG	3540
25	CTGGGGGCA AGGCTTTTA CTACAGAG GAACAGGCC TGGCTTGG GGGGGGGG	3600
	TCTTCAGAT TTCTGGCTT GAGGTTCAC TACACACC CACTGGGAT AACAGGGCG	3660
30	CGGACTCTT CGGCATCG CTTGTACTAC ACGCTGGC TGGGGGCTT GAGCGGGC	3720
	ATCATGGAGC TGGGCTGGC GTACAGGCC GTGATGGCA TCGGGGCA GAGAGGGC	3780
	TTGTCTCA CGGCTACTG CAGGACAAG TGCACCGC TGGGCTGC CGCTCAGG	3840
35	ATTACATCT TGGCTCTCA GCTCCACAG CACCTAGG GGGGAGGT GTTCACGTG	3900
	CTGGGAGGG AGGGGGGA GACAGATC GTGACAGG ACAACACTA CAGGCCAC	3960
40	TTCCAGGAGA TCGCATGTT GAAGAAGTC GTGTCTGTC AGCGGGGA CGTCTCATC	4020
	AACCTTGA CATAACAC GAAGACAG AGGCTGGCA CGGGGGGG CTGGGATC	4080
	CTGGAGGAGA TGTGGTCAA CTAATGCAC TACTACCCC AGAGCAGCT GGAGCTCTG	4140
45	AAGAGGGCG TGGAGCTG CTCTCTAC AAGTACTTC GCTGTGGA CAGGTTCAC	4200

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AGGAGGAG TCTGCACCTG CCCCCAGGG TCTGTCCCTG AGCAGTTTGC CTCGGTGGCC 4260
 TGGAACTCCT TCAACCGGA GGTGCTCAAG GGGCTGTAGG GCTTCGGACC CATCTCCATG 4320
 5 CACTGCAACA GGTCTCGGC CGTCCCTTC CAGGGGAGT GGAATGCGA GGGCTGGCT 4380
 GAGATGGTGT CCAGGTGGA AGAGCCACC CCTCACTGC CAGCCAGCA GGCTCAGAGC 4440
 CCGGCGGCGC CCACGGTGT GACATCAGT GGGGCAAG GCTGAAGTG GCGGCGGC 4499

10

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

25

(vii) IMMEDIATE SOURCE:
 (B) CLONE: oIRES-074

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AAAGGGGGG CCGCTCTGCC TCCCCCCCC

30

35 (2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

45

(iv) ANTI-SENSE: NO

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- (vii) IMMEDIATE SOURCE:
(B) CLONE: oZeocin-077

5

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AAACTGGAGT CAGTCTGCT CCTGGGCAC

30

- 10 (2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15

- (ii) MOLECULE TYPE: cDNA

- 20 (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- 25 (vii) IMMEDIATE SOURCE:
(B) CLONE: OIRES-Zeocin-075

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

30

GGTCAACTTG GGCATGGTTC TGGCAAGCTT

30

- (2) INFORMATION FOR SEQ ID NO:27:

- 35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- 45 (iv) ANTI-SENSE: NO

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(vii) IMMEDIATE SOURCE:
(B) CLONE: oIRES-Zeocin-076

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTTGCCACAA CCATGGCCAA GTTGACAGT

30

(2) INFORMATION FOR SEQ ID NO:28:

10

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5540 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

20

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

25

(B) CLONE: POMDACTH-IRES-THD-IRES-DEH-IRES-Zeocin

(ix) FEATURE:

(A) NAME/KEY: 5'UTR
(B) LOCATION: 1..118

30

(ix) FEATURE:

(A) NAME/KEY: exon
(B) LOCATION: 119..164

35

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 165..243

(ix) FEATURE:

40

(A) NAME/KEY: exon
(B) LOCATION: 244..557

(ix) FEATURE:

45

(A) NAME/KEY: intron
(B) LOCATION: 558..1155

(ix) FEATURE:

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(A) NAME/KEY: exon
(B) LOCATION: 1156..2166

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2167..2766

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 2767..4560

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 4561..5159

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 5160..5534

(ix) FEATURE:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 5535..5540

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

25	AGGCTGGTA CCGAGCTGG ATCCACTAGT AACGGGCGC AGTGTCCTGG AATTCCTGAG	60
	ATATCCATCA CACTGGGGC CGGTCACCC CTAGAGTGA GCCTGACGG TCTTACAAAT	120
30	GAAATGCAGC TGGGTATCT TCTTCTGAT GGCAGTGGT ACAGGTAAGG GGTCCCAAG	180
	TCCAAACTT GAGGGTCCAT AACTCTGTG ACAGTGGCA TACTTTGOC TTCTTTCTA	240
35	CAGGGGIGAA TTGGCTTTC CCGGCTTCC CCGTGAGTT CAAGAGGGAG CTGACTGGC	300
	AGGACTGCG GGAGGGAGAT GGGGCGAGG GCGTGGGA TGAAGGGCA GGGGCGAGG	360
	CGACTTGA GCACAGCTG CTGGTGGGG CAGAGAGAA GGCAGGGC CCGTACAGA	420
40	TGAGCACTT CCGCTGGGC AGGCGGCA AGGACAGG CTAGGGGGT TTCATGAAT	480
	CGAGAGAG CCAGAGGCC CTGGTGGC TGTTCAAAA CGCATCATC AAGAGGCT	540
45	ACAGAGGG CAGTGAGG CACAGGGC CCGTCTGCT CCGGGGGG TACGTTACT	600
	GGCGAGGC GCTTGAATA AGGCGGTGT GCGTTGTCT ATATGTATT TTCCACATA	660

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	TTGGGTCCTT TTGGCAATGT GAGGGGCGGG AAACCTGGGC CTGCTTCTTT GAGGAGCATT	720
	OCTAGGGGTC TTTCOOCTCT CGCCAAAGGA ATGCAAGGTC TGTGAATGT CGGAAGGAA	780
5	CGAGTTCCTC TGGAGCTTC TTGAAGACAA ACAAGTCTG TAGGAAOCT TTGCAGGCAG	840
	CGGAACCCOC CAOCTGGGA CAGGTGCTC TGCGGCCAAA AGCCAGTGT ATAAGATACA	900
10	OCTGCAAGG CGGCACAACC CCAGTGCAC GTTGIGAGIT GGTAGTGT GGAAGAGTC	960
	AAATGGCTCT CCTCAGGT ATTCACAAG GGGCTGAGG ATGCCCAGAA GSTACCCAT	1020
	TGTATGGGAT CTGATCTGG GCTCGGTGC ACATGCTTTA CATGIGTTA GTGAGGTTA	1080
15	AAAAAGTCT AGGCCCCCG AACACGGGG AGTGGTTTT CTTTGAAA ACACATGAT	1140
	AAGCTTGCA CAACTAGGT CCGCTGTTT CCAGAAAG TGTCGTAAT GGCAGTGT	1200
20	CACACCTGG TCACCAAGT TGACCTGAT CTGACCTGG ACCACCGGG CTCTCTGAC	1260
	CAGGIGTATC GGCAGGTGG GAGCTGATT GCAGATIG CTTCCAGTA CAGCAGGT	1320
	GACCAATTC CCACTGGA ATACACAGG GAGAGATIG CTAOCTGGA GGAGTATAT	1380
25	GTCAOCTGA AGGCTCTTA TGCTACCAT GCTGCGGG AGCACCTGA GGGTTCCAG	1440
	CTCTGGAC GGTACTGIG CTAOCAGAG GACAGATCC CACAGCTGA GGAAGTGC	1500
30	CGCTCTTGA AGGAGGGAC TGGCTCCAG CTGGACCG TGCGGGTCT ACTGTCCGC	1560
	CGTATTTTC TGGCAGTCT GGCCTCCGC GTGTTCAAT GCACCCAGTA TATCCGCAT	1620
	GCTCTCAC CTATGATTC AACTGAGCG GACTGCTGC ATGAGCTGT GGCATGTA	1680
35	CCATGTTGG CTGACCGAC ATTGCGCAG TTCTCCAGG ACATGGACT TGCATCTG	1740
	GGGCTCAG ATGAGAAT TGAAATCT TCACGGGT ACTGGTTCAC TGGAATTC	1800
40	GGCTATGTA AACAGATGG GGAGCTGAG GCTTATGGT CAGGCTGCT GTCTTCTAC	1860
	GGAGGCTOC TGCCTOCT GTACAGGAG OCTGAGTCC GAGCTTTGA CCAAGACA	1920
	GCAGCTGTC AGCCTACA AGATCAACC TACAGCTG TGTACTTTGT GTGAGAGC	1980
45	TTCATGAG CCAAGACAA GCTAGGAC TATGCTCTC GTATCCAGG CCAATCTCT	2040

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	GTGAGTTTG ACOOGTACAC ACTGGGCATT GACGTACTGG ACAGCOCTCA CACCATCCAG	2100
	CGCTOCTTGG AGGGGGTCCA GGATGAGCTG CACACCTTGS CCCAGGACT GAGTGCCATT	2160
5	AGCTAAATGC ATAGGATCCG CCCCCTCCOC TCCCCCCCC CTAAGGTAC TGGGCGAGC	2220
	CGCTTGGAT AAGGCGGGTG TGGTTTGTG TATAGTAT TTTCCACAT ATTGCGGTCT	2280
	TTTGGCAATG TGAGGGGCG GAAOCTGGC CCGTCTTCT TGAAGACAT TCTTAGGGT	2340
10	CTTTCCCTC TGGCAAGG AATGCAAGT CTGTGAATG TGTGAGGA AGCAGTCT	2400
	CTGGAAGCTT CTGAAGACA AACAGGTCT GTAGGACCC TTTCAGGCA GCGGACCC	2460
15	CCOCTGGCG ACAGGTGCT CTGGGCGCA AAGCAAGTG TATAAGATC AACTGCAAG	2520
	GCGGCACAC CCCAGTCCA CGTTGTGAGT TGGATAGTG TGAAGAGT CAAATGGCTC	2580
	TCTCAGCG TATTCAACA GGGCTGAG GATGCGAGA AGGTACCCA TTGTATGGA	2640
20	TCGTATCGG GGCCTGGTG CACATGCTT ACAATGTGT AGTCAGGTT AAAAAAGTC	2700
	TAGGCCCCC GAACAGGG GAGTGGTTT TCTTTGAAA AACAGATGA TAGCTTGC	2760
25	ACAACCATGT AGGCACCGC GGTCGGGTC TTCTGGTCA TCTGTGGC TGCATGCG	2820
	GGCTGGGTC CCGGGGAG CCCCCTCCOC TTCCACATC CCGTGAAC CAGGGGAC	2880
	CTGAGCTGT CCGGACAT CAGCTATCG CAGGAGACA TCTACTTCA GCTCTGGTG	2940
30	CGGAGCTCA AGCTGGTGT CCGTTTGGG ATGTGGGAC GAGGGGACT GGAGATGCT	3000
	GACTGGTGG TCTCTGGAC TGACAGGGC GGGGCTACT TTGGGGATC CTGGAGTAC	3060
35	CAGAGGGGC AGGTCCACT GGACTCCAG CAGGATTAC AGCTTCTGG GGCACAGG	3120
	ACTCCAGAG GCTGTACT GCTCTTCAAG AGGCTTTTG GCACTGTA CCCCACGAC	3180
	TACTCATG AGGAGGCAC CGTCCACTG GTGTATGGT TCTGGAGGA GCGCTCCG	3240
40	TGCTGGAGT CCATCAACAC ATCCGCTTG CACAGGGGC TGACAGGGT GCAGCTGCT	3300
	AAGCCAGCA TCCCAAGC GGGGCTGCC GCGGACGC GCAACATGA GATCCGCG	3360
45	CCGAGCTC TCATCCCGG CAGGAGAC AGGTACTGT GCTAGTAC CAGCTCCG	3420
	GAGGCTTC CCGGCACA CATGTGATG TAGAGCCA TGTACCGA GGCACAG	3480

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	GGGCTGGTGC ACCACATGGA GGTCTTCCAG TGGGCGCGCG AGTTCGAGAC CATCCCCAC	3540
	TTTAGCGGGC CCTGGGACTC CAAGATGAAG CCGTAGGGC TCAACTTCTG CCGTCAGTG	3600
5	CTGGCGGCT GGGGCGGG GGGCAGGC TTTTACTACC CAGAGGAGC AGGCGTGGC	3660
	TTGGGGGGC CCGGCTCTC CAGATTCTC CGCTGGAG TTTACTACA CACCCACTG	3720
10	GTCATAACAG GCGGCGGGA CTCTGGGC ATCGCGCTGT ACTACAGGC TGGCGTGG	3780
	CGCTTCAGC CCGGCATCAT GGAGCGGC CTGGGTACA CGCGCGAT GGCATCCCC	3840
	CGCAGGAGA CCGGCTTGT CTTACAGGC TACGTAGCG ACAAGTGC CAGCTGGC	3900
15	CTCGCGGCT CAGGGATCA CATCTGGC TCTAGCTC ACAGCACT GACGCGGG	3960
	AAGGTGGTA CAGTCTGGC CAGGAGGC CGGAGACAG AGATGTGAA CAGGACAC	4020
20	CATACAGC CACACTTCA GGAGTCCG ATGTGAGA AGGTGTGT TGTCCAGCG	4080
	GGAGGTGC TCATCACTC TTGCATAC AACCGGAG ACAGGAGCT GGCACGGT	4140
	GGGGCTTGG GATCTGGA GGAGTGTG GTCACTATG TGCCTACTA CCGCAGCG	4200
25	CAGCTGGAG TCTGCAAG CCGGTGGC CTTGGCTC TGCACAGTA CTTCGGCTC	4260
	GTCACAGT TCACAGGA GGAGTGTG ACGTCCCC AGCGTCTGT CCGTGAAG	4320
30	TTTGGCTCG TGGCTGGA CTCTTCAAC CGGAGGTG TCAGGCGT GTAGGCTT	4380
	GCACCATCT CCATGACTG CACAGGTG TGGCGGTG GCTTCAGG CGAGTGAAT	4440
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35	AGCAGGCTC AGAGCGGC CGCGGCAAC GTCTGAACA TCAGTGGGG CAAGGCTGA	4560
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40	AATAGGCG GGTGGGTT GTCATATG TATTTTCAC CATATTGG TCTTTTGGC	4680
	ATGTAGGC CCGGAACT GCGCTGCT TCTTACAG CATTCAGG GGTCTTCC	4740
	CTCTGCGAA AGGAATCAA GGTCTGTA ATGTGTGA GGAGCAGT CCTCTGAG	4800
45	CTTCTGAG ACAACACG TCTGTAGA CCGTTCAG GTAGGGAC CCGCACTG	4860

- 88 -

GGGACAGGTC CCCTCGGGC CAAAGCCAC GGTATAGA TACACCTGA AAGGGGCAC 4920
 AAAAAAGTC CCAAGTGTG AGTTGGATG TTGGGAAAG AGTCAATGG CTCTCTCAA 4980
 5 GGGTATCAA CAAAGGGCTG AAGGATGOC AGAAGGTACC CCATTGTATG GGATCTGATC 5040
 TGGGGGCTCG GTCACATGC TTTACATGTC TTTAGTGTG GTTAAAAAC GTCTAGGOC 5100
 CCGAACCAC GGGGAGTGG TTTTCTTTG AAAACAGA TGATAGCTT GGCACACA 5160
 10 TGGCCAGTT GACAGTGC GTTGGGTG TCACGGGG CAGGTGOC GGAGGGTGC 5220
 AGTCTGGAC CAGCGCTC GGGTCTGC GGGACTTGT GGAGAGAC TTGGGGTG 5280
 15 TGGTGGGA CAGGTGAC CTGTATCA GGGGGTCA GGACAGGTC GTGGGACA 5340
 AAAAACTG CTGGGTG GTGGGGTC TGAAGACT GTAGGGAG TGGTGGAG 5400
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 20 CGTGGGGG GGAGTGC CTGGGAC CGGGGCA CTGGGTGAC TTGTGGG 5520
 AGGAGTGA CTACTGAG 5540

25 (2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 829 base pairs
 (B) TYPE: nucleic acid
 30 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40 (vii) IMMEDIATE SOURCE:
 (B) CLONE: ProAKS

(ix) FEATURE:
 (A) NAME/KEY: 5'UTR
 45 (B) LOCATION: 1..16

(ix) FEATURE:

SUBSTITUTE SHEET (RULE 26)

- 89 -

(A) NAME/KEY: exon
(B) LOCATION: 17..820

(ix) FEATURE:

5 (A) NAME/KEY: 3'UTR
(B) LOCATION: 821..829

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

10 CCCAGCTTC GGCACCATGG CCGGGTTCCT GACACTTTC ACTTGGCTGC TGTTCCTGG 60
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 15 CCGCTAGTG CCGCGGGG ACATCACTT CCTGGCTTC GTAATGGAAT GGAAGGTAA 180
 ACTGCTTCT CTGAAATTT GGAAGCTG CAGGAGCTC CTGCAGCTG CCAACACGA 240
 GCTTCTCAA GATGGACCA GCACTTCAG AGAAATAGC AAGCGGAG AAGGCATTT 300
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 30 AAGCCCCAA CTGAAGATG AAGCCAGA GCTGCAGAG CGATATGGG GCTTCATG 660
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 35 GCGCTTGGC GAGCTTCG CTTCCAGA AGAGGGGA AGTACTCA AAGAGTTC 780
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(2) INFORMATION FOR SEQ ID NO:30:

40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 598 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 45 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- 90 -

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

5

(vii) IMMEDIATE SOURCE:

(B) CLONE: IRES sequence

10

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 1..598

15

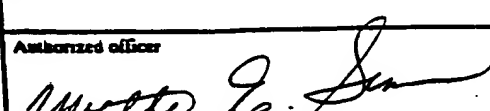
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

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GCCAAAGGAA TGCAAGGTCT GTTGAAATGTC GCGAGGAAG CAGTCTCTCT GGAGCTTCT	240
25 TGAAGACAAA CAAGTCTGT AGGACCTT TCCAGGCAGC GGAACCCCC AACCTGGGAC	300
AGGTGCTCTT GCGGCAAAA GCGAGGIGTA TAGATACAC CTGCAAGGC GGCACAACTC	360
CAGTGGCAGG TTGCGAGTTG GATAGTTGIG GAAAGAGTCA AATGGCTCTC CTCAGGCTA	420
30 TTCAACAAGG GGCTGAGGA TGGCCAGAG GTACCCCATTT GTATGGGATC TGAICTGGGG	480
CCTGGGIGCA CATGCTTTAC ATGIGTTTAG TCGAGGTAA AAAAGCTCTA GGGCCCCGGA	540
35 AACACGGGGA CGTGGTTTTC CTTTGAAAA CAGATGATA AGCTTGGCAC AACCATGG	598

Applicant's or agent's file reference number	CTI/29 CIP PCT	International application No.
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>54</u> line <u>14-23</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Identification Reference by Depositor: <u>Cell Line, RINa/ProA/P030/P088</u>	
Date of deposit 07 June 1995 (07.06.95)	Accession Number CRL 11921
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input checked="" type="checkbox"/>	
In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by requester (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
EPO	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer 	Authorized officer

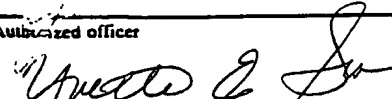
SUBSTITUTE SHEET (RULE 26)

90/2

Applicant's or agent's file reference number	CTI/29 CIP PCT	International application No.
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>54</u> , line <u>S</u> <u>14-23</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depository institution American Type Culture Collection	
Address of depository institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Identification Reference by Depositor: P030/P088	
Date of deposit 07 June 1995 (07.06.95)	Accession Number CRL 11921
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input checked="" type="checkbox"/>	
In respect of the designation of Finland, until the application has been laid open to public inspection by the Finnish Patent Office, or has been finally decided upon by the Finnish Patent Office without having been laid open to public inspection, samples of the deposited microorganisms will be made available only to an expert in the art.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
Finland	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer 	Authorized officer

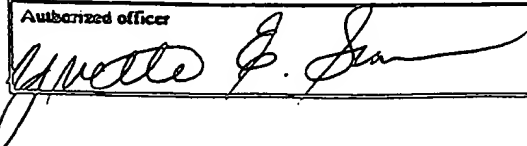
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90/3

Applicant's or agent's file reference number	CTI/29 CIP PCT	International application No.
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>54</u> line <u>S 14-23</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Identification Reference by Depositor: <u>Cell Line, RINa/ProA/ P030/P088</u>	
Date of deposit 07 June 1995 (07.06.95)	Accession Number CRL 11921
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
Applicant(s) hereby give notice of my/our intention that samples of the above-identified culture shall be available only to experts in accordance with paragraph 3 of the Fourth Schedule to the Patents Rules 1995.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
Singapore	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
For receiving Office use only <input checked="" type="checkbox"/> This sheet was received with the international application Authorized officer 	For International Bureau use only <input type="checkbox"/> This sheet was received by the International Bureau on: Authorized officer

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WE CLAIM:

1. A cell stably transformed to produce at least one analgesic compound from each of the groups consisting of endorphins, enkephalins, and catecholamines.
2. The cell of claim 1, wherein the endorphin is β -endorphin.
3. The cell of claim 1, wherein the enkephalin is met-enkephalin.
4. The cell of claim 1, wherein the catecholamine is norepinephrine or epinephrine.
5. The cell of any one of claims 1-4 wherein the cell is a RIN cell.
6. The cell of any one of claims 1-4 wherein the cell is an AtT-20 cell.
7. The cell of any one of claims 1-6 wherein the cell additionally produces a compound selected from the group consisting of galanin, somatostatin, neuropeptide Y, neurotensin, or cholecystokinin.
8. A cell transformed with a DNA encoding POMC, a DNA encoding TH, a DNA encoding DBH, and a DNA encoding ProA, each DNA molecule operably linked to an expression control sequence.

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9. The cell of claim 8 wherein the cell is transformed with pCEP4-POMC-030, pcDNA3-hproA+KS-091, and pZeo-pCMV-rTHAKS-IRES-bDBH-088.

10. The cell of claim 8 wherein the cell is transformed with pCEP4-h POMC- Δ ACTH-032, pBS-CMV-proA, and pZeo-pCMV-rTHAKS-IRES-bDBH-088.

11. The cell of claim 8 wherein the cell is transformed with pcDNA3-hPOMC Δ ACTH-IRES-rTHD-IRES-bDBH-IRES-Zeocin-073 and pcDNA3-proA+KS-091.

12. A transformed cell producing at least one enkephalin, one endorphin and one catecholamine, wherein the cell is transformed with:

a first vector containing a DNA encoding POMC operably linked to an expression control sequence,

a second vector containing a DNA encoding pro-enkephalin A operably linked to an expression control sequence,

a third vector containing a DNA encoding TH operably linked to an expression control sequence and a DNA encoding dopamine beta hydroxylase operably linked to an expression control sequence.

13. A method for treating pain comprising implanting at an implantation site in a patient a therapeutically effective number of the cells of any of claims 1-12.

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14. The method of claim 13 wherein the cells are encapsulated in a semi-permeable membrane to form a bioartificial organ.

15. The method of claim 14 wherein the bioartificial organ is immunoisulatory.

16. The method of any one of claims 13-15 wherein the implantation site is the CNS.

17. The method of any one of claims 13-15 wherein the implantation site is the sub-arachnoid space.

18. A method of producing a cell that secretes at least one enkephalin, one endorphin and one catecholamine, comprising transforming the cell with a DNA encoding POMC operably linked to a first expression control sequence, a DNA encoding pro-enkephalin A operably linked to a second expression control sequence, and a DNA encoding TH operably linked to a third expression control sequence and a DNA encoding dopamine beta hydroxylase operably linked to a fourth expression control sequence.

19. The method of claim 18 wherein said first, second, third and fourth expression control sequences are identical.

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20. The use of the cells of any of claims 1-12 to manufacture a medicant for treatment of pain.

21. The cells of claim 20 wherein the cells are implanted.

22. The cells of any one of claims 21-22 wherein the cells are encapsulated in a semi-permeable membrane to form a bioartificial organ.

23. The cells of claim 22 wherein the bioartificial organ is immunoisulatory.

24. The cells of any one of claims 21-23 wherein the implantation site is the CNS.

25. The cells of any one of claims 21-23 wherein the implantation site is the sub-arachnoid space.

26. A bioartificial organ comprising:

(a) a biocompatible, permeable jacket surrounding a core; and

(b) said core comprising at least one living cell transformed to produce at least one analgesic compound from each of the groups consisting of endorphins, enkephalins, and catecholamines.

27. The bioartificial organ of claim 26 for use in treating pain.

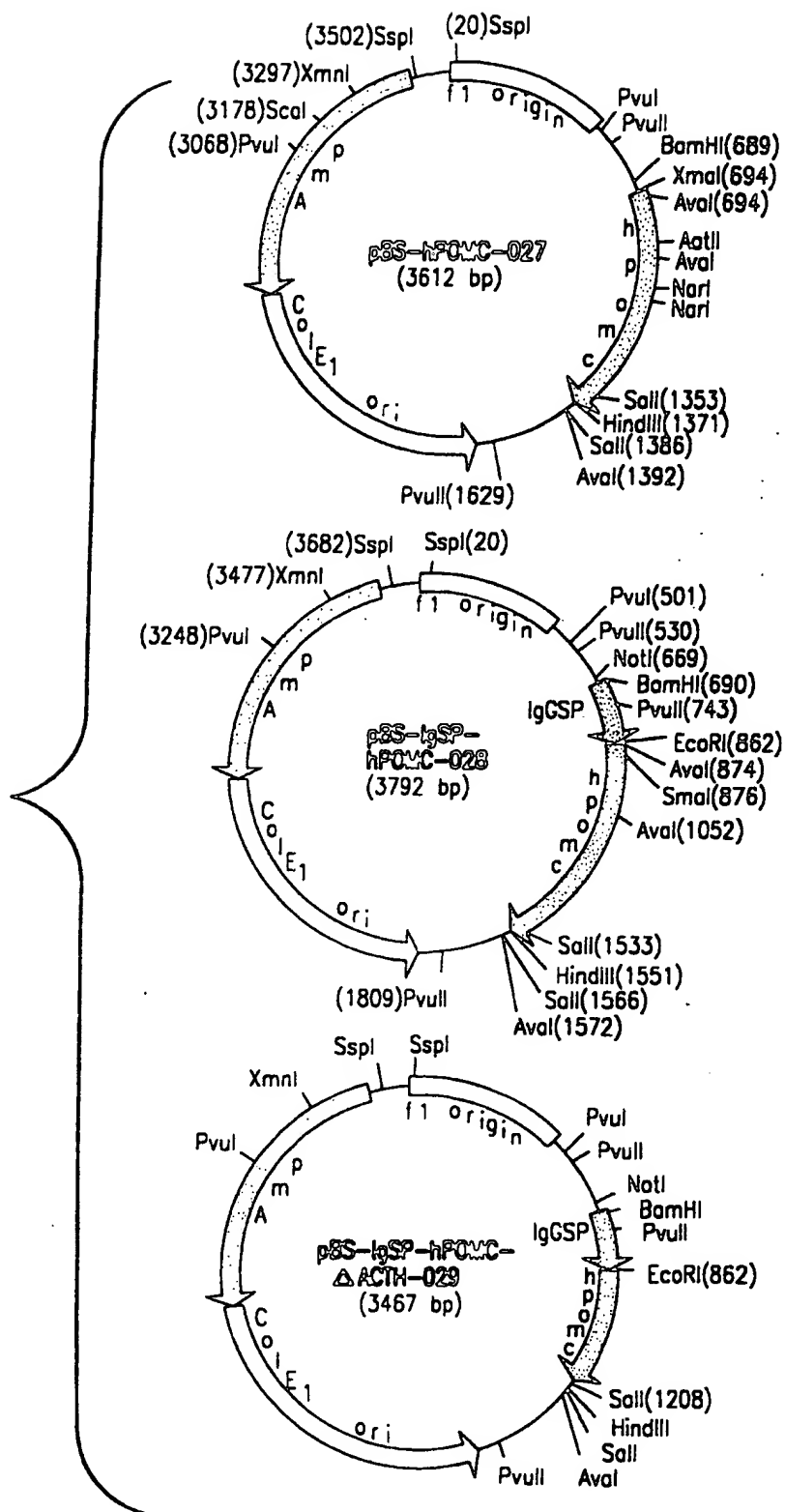
- 95 -

28. A method of making a bioartificial organ comprising encapsulating a core comprising at least one living cell transformed to produce at least one analgesic compound from each of the groups consisting of endorphins, enkephalins, and catecholamines, with a biocompatible, permeable jacket.

29. The use of a bioartificial organ comprising the cells of claims 1-12 in manufacture of a medicament for treating of pain.

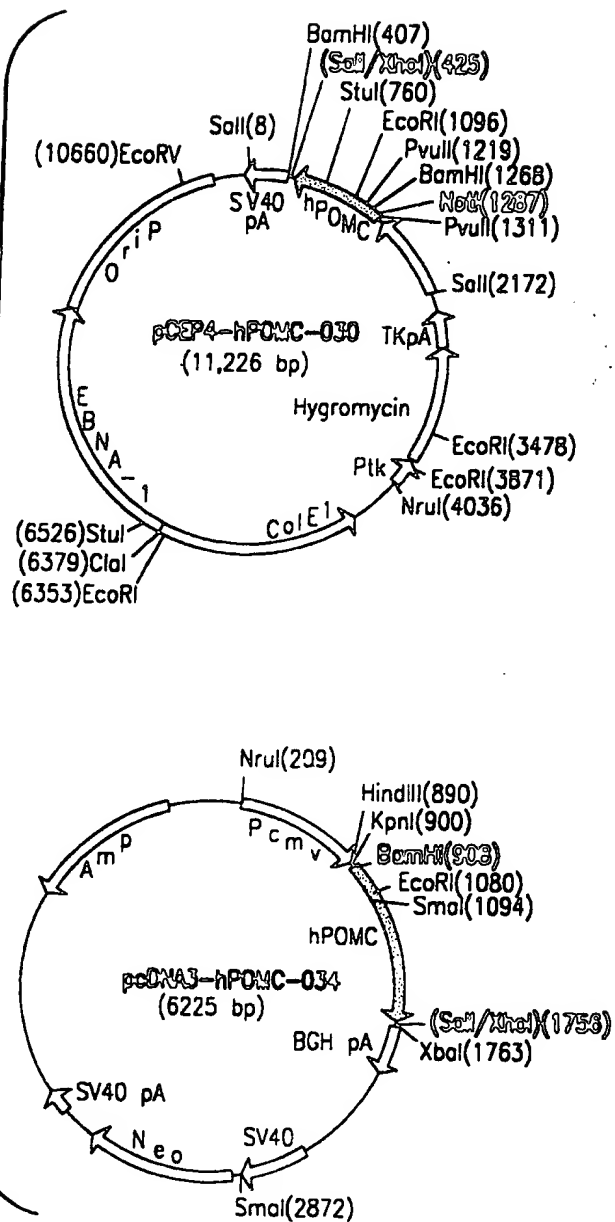
1 / 13

FIG. 1



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FIG. 2a



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FIG. 2b

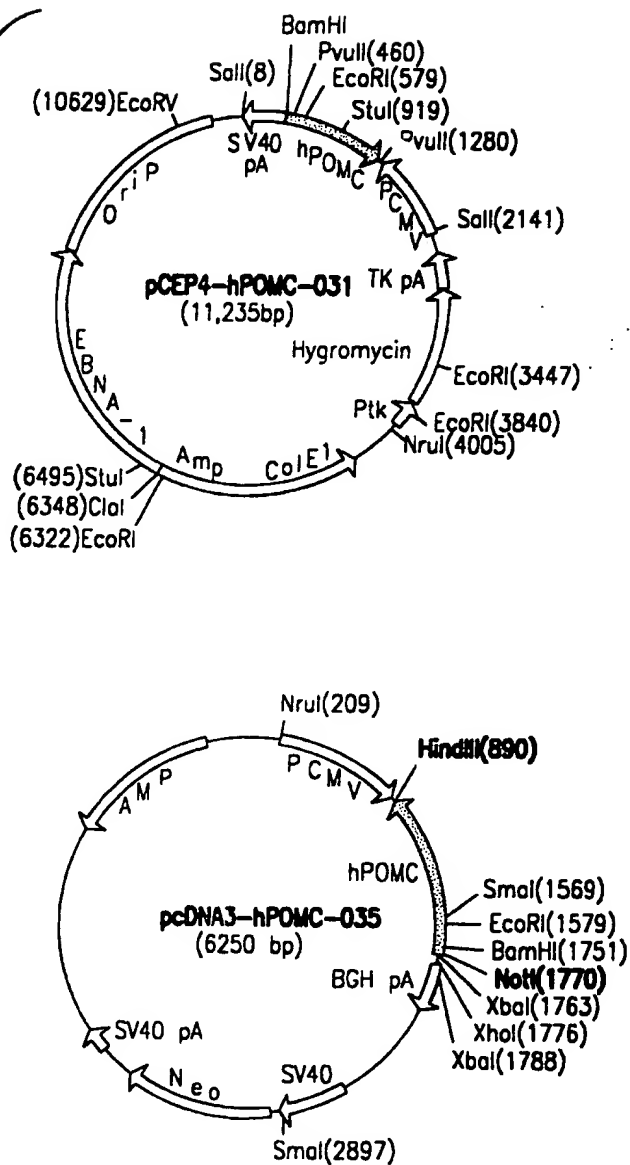
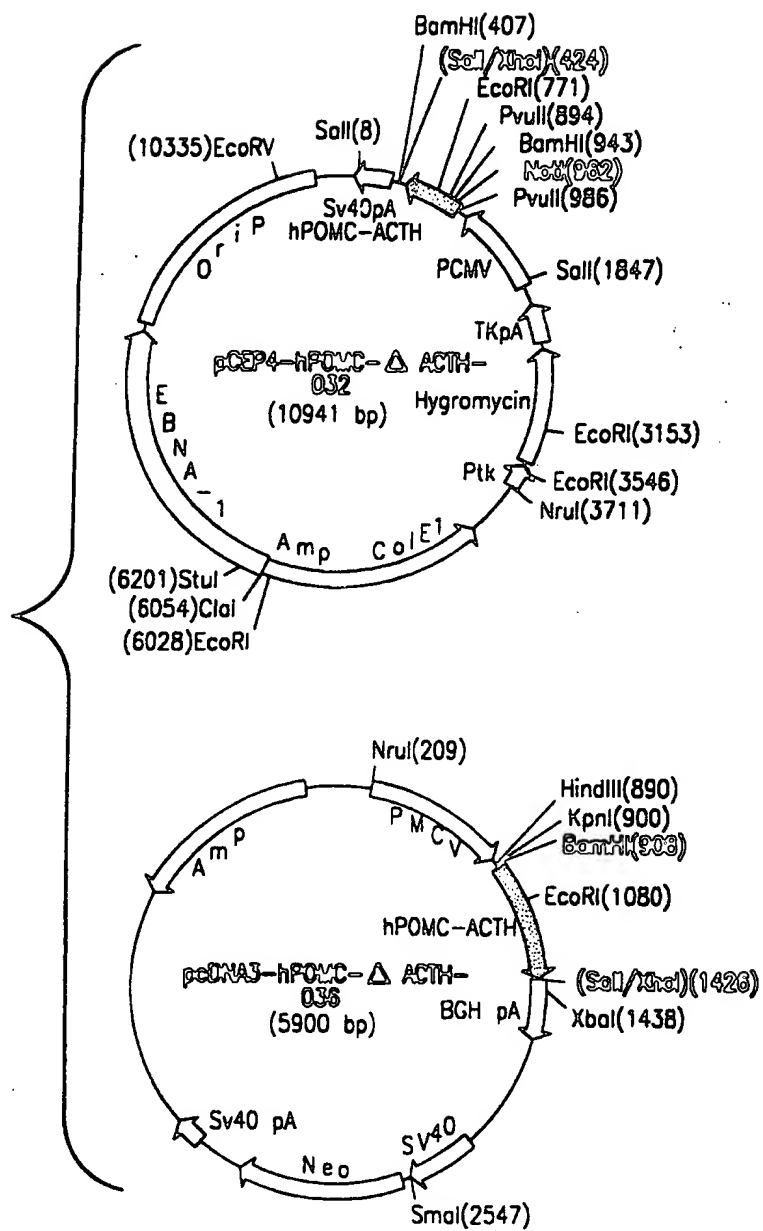
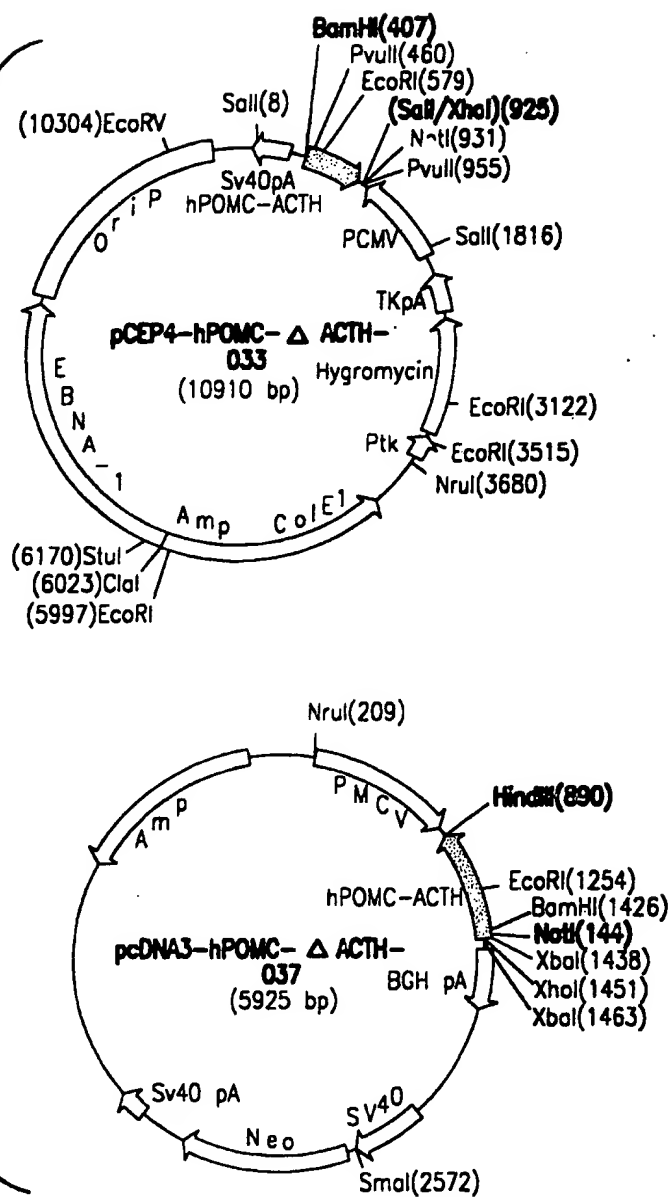


FIG. 3a



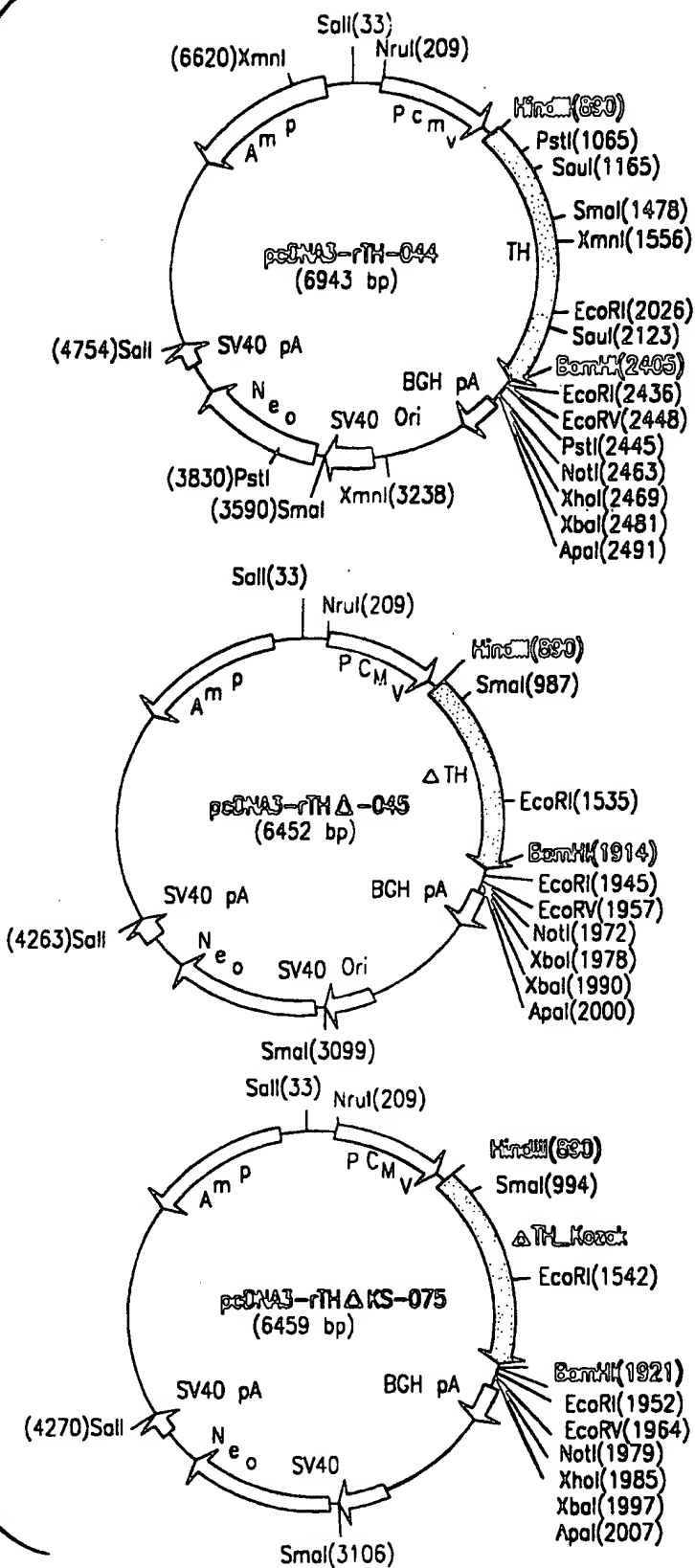
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FIG. 3b



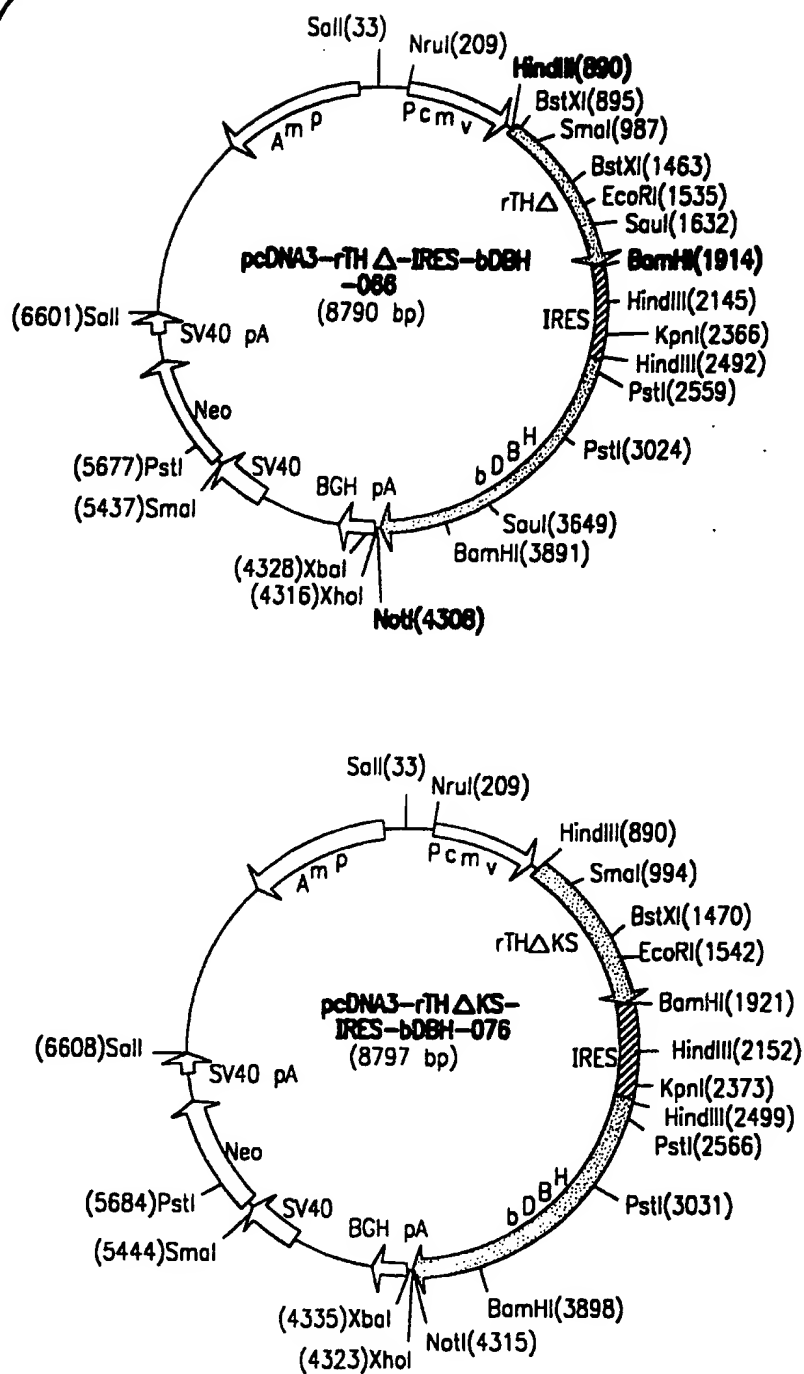
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FIG. 4



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FIG. 5



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FIG. 6

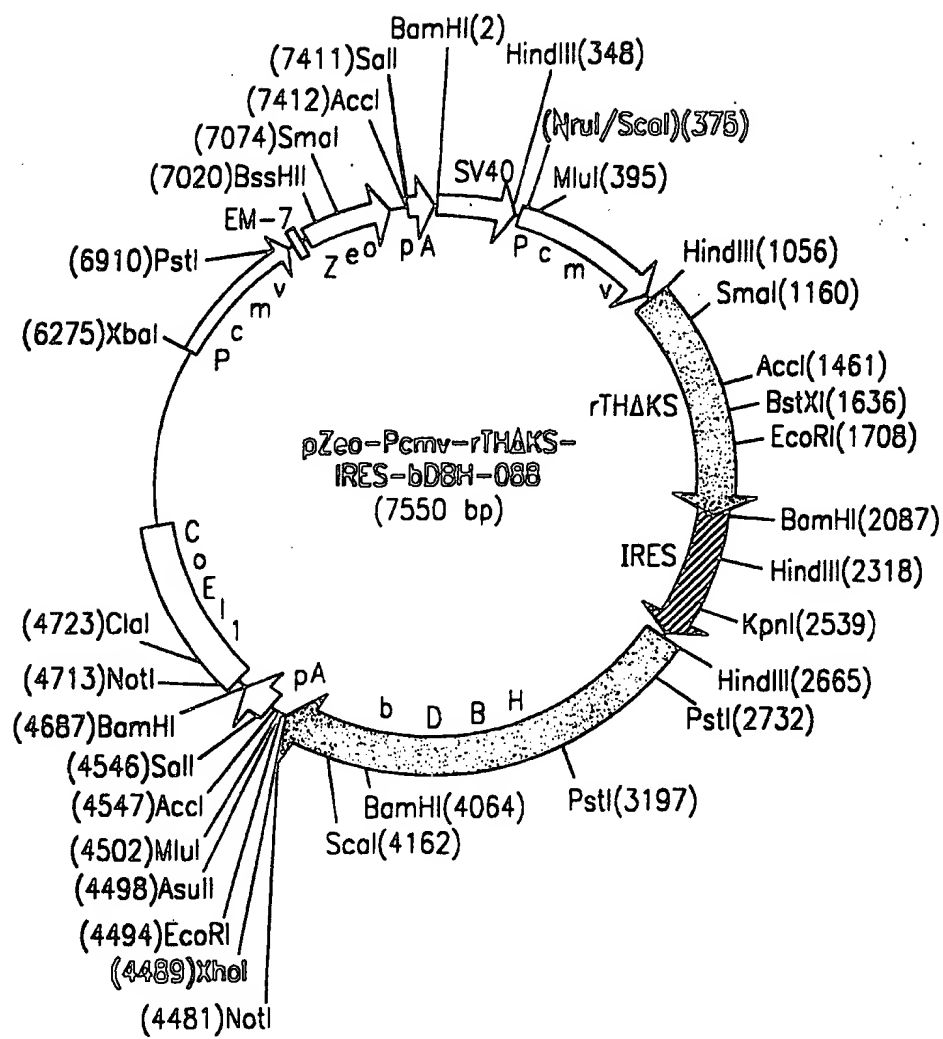
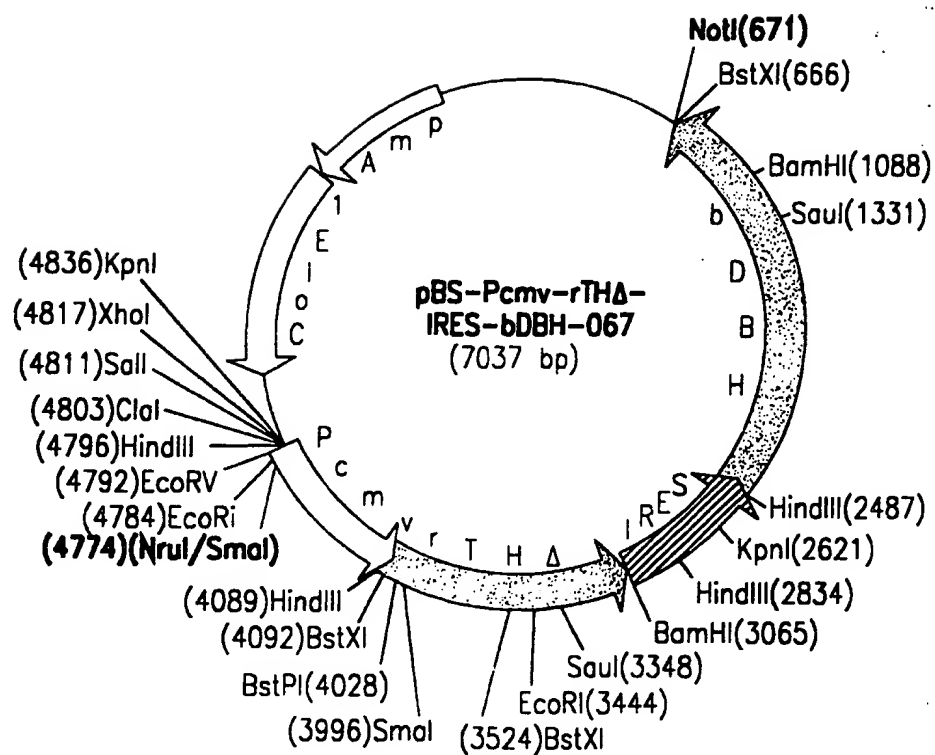
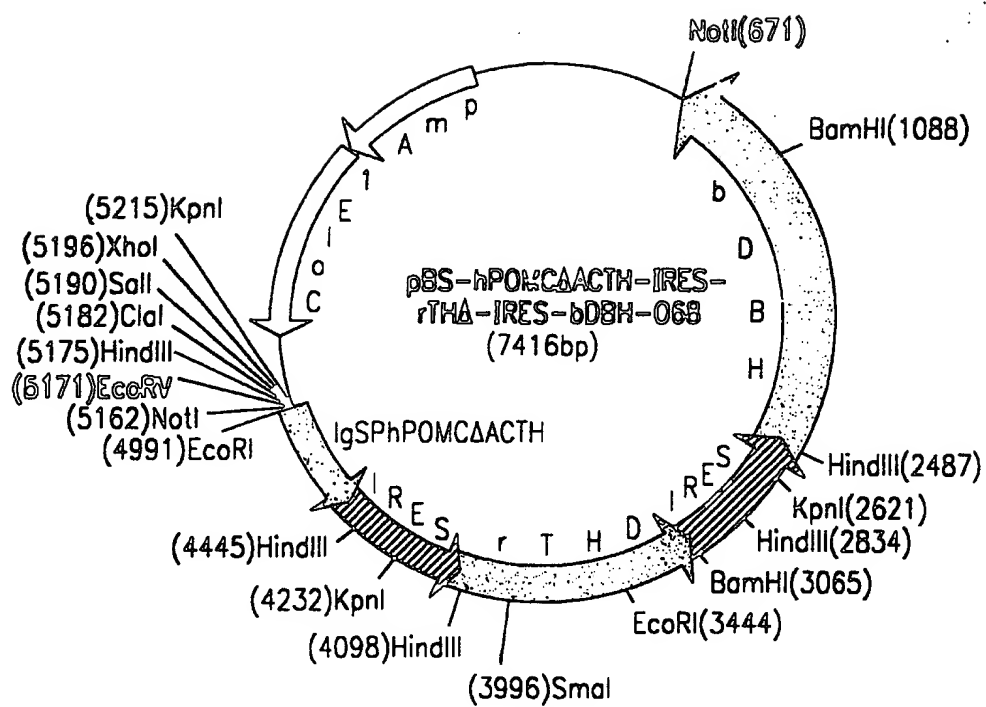


FIG. 7



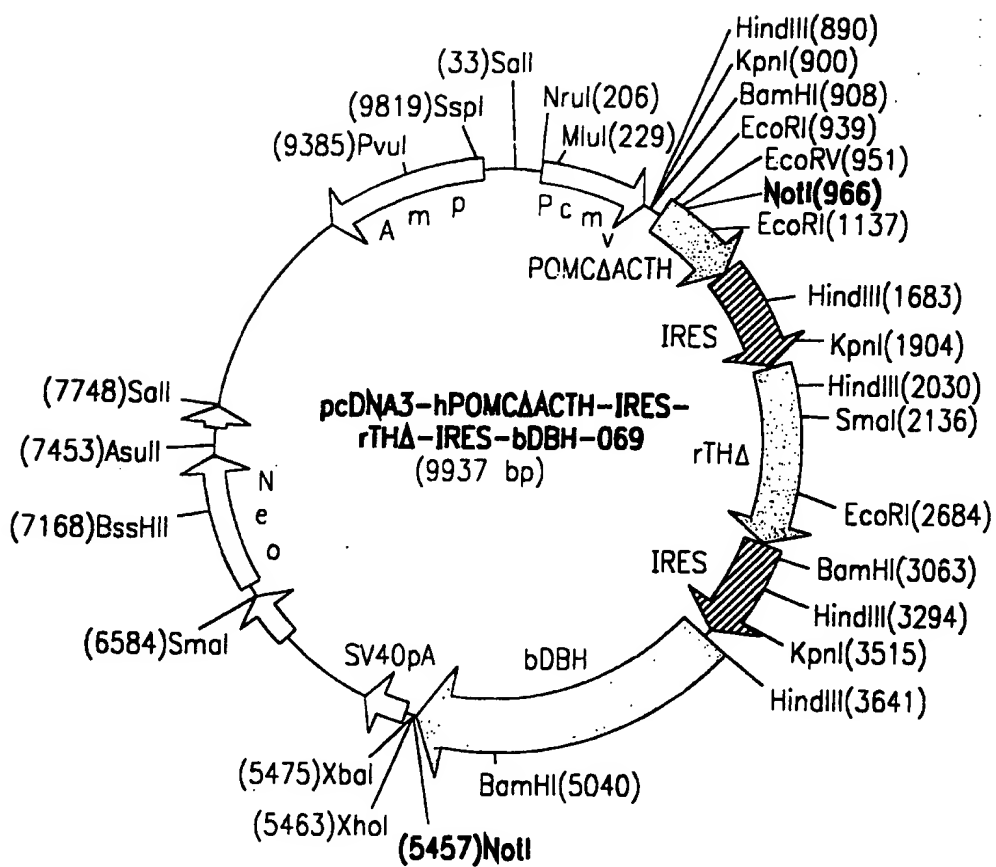
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FIG. 8



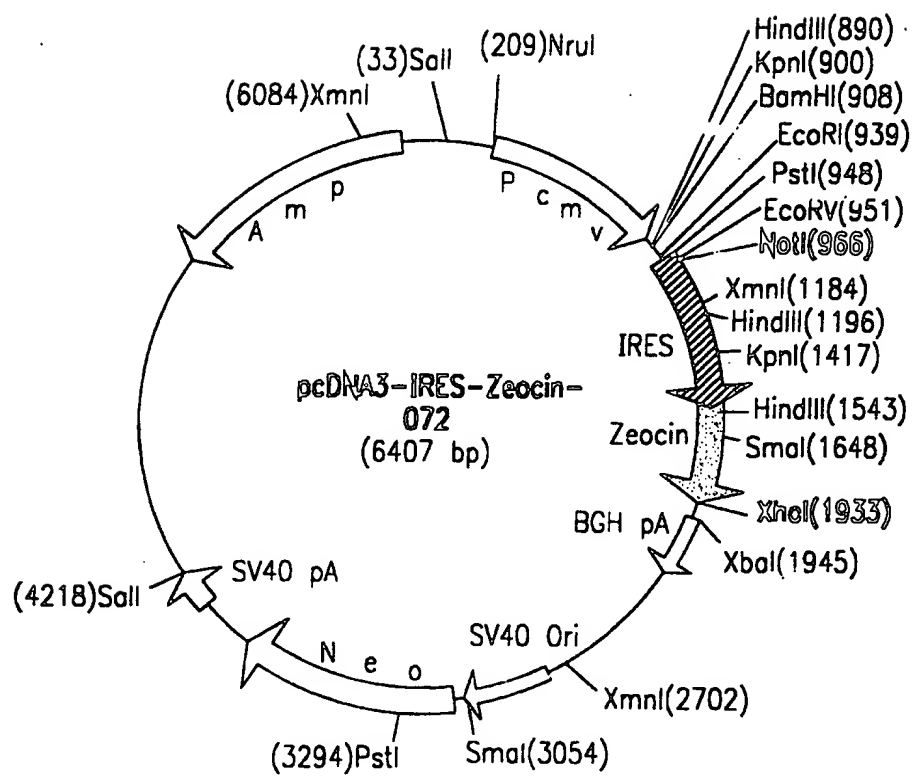
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FIG. 9



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FIG. 10



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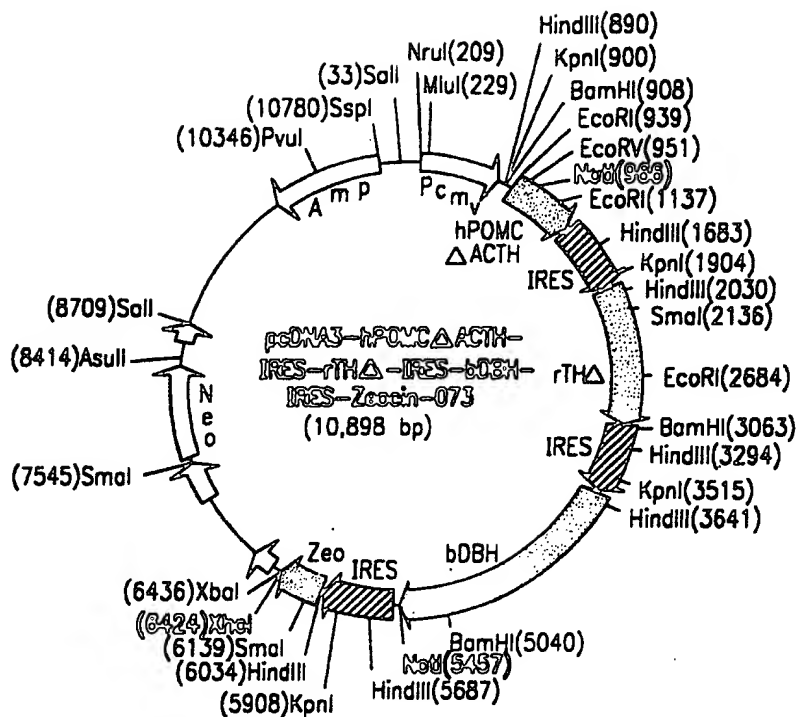


FIG. 11

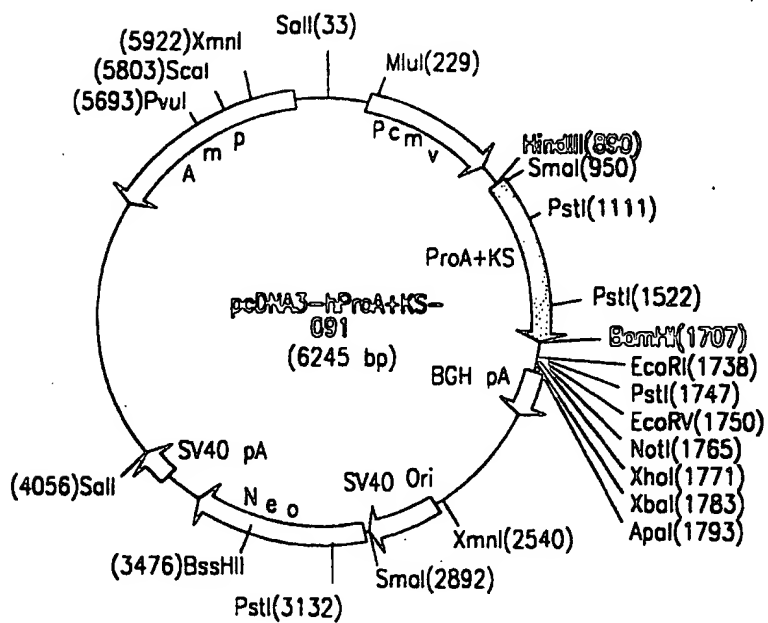


FIG. 12

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/09629

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/87 C12N5/10 A61K9/48 A61K38/16 A61K38/33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,95 05452 (CYTOTHERAPEUTICS, INC.) 23 February 1995 see the whole document, especially pages 12-31 and Example 6. ---	1-4,8, 12-29
A	J. NEUROSCI., vol. 14, 1994, pages 4806-4814, XP002018157 H.H. WU ET AL.: "Implantation of AtT-20 or genetically modified AtT-20/hENK cells in mouse spinal cord induced antinociception and opioid tolerance" cited in the application see the discussion. --- -/--	1

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * & * document member of the same patent family

Date of the actual completion of the international search

14 November 1996

Date of mailing of the international search report

28.11.96

Name and mailing address of the ISA

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Authorized officer

Yeats, S

INTERNATIONAL SEARCH REPORT

In International Application No

PCT/US 96/09629

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PROC. NATL. ACAD. SCI. USA, vol. 83, 1986, pages 7522-7526, XP002018158 J. SAGEN ET AL.: "Analgesia induced by isolated bovine chromaffin cells implanted in rat spinal cord" cited in the application see the abstract and discussion.</p> <p style="text-align: center;">---</p>	1
A	<p>NATURE, vol. 297, 1982, pages 335-339, XP002018159 M. COCHET ET AL.: "Characterization of the structural gene and putative 5'-regulatory sequences for human proopiomelanocortin" cited in the application see the whole document.</p> <p style="text-align: center;">-----</p>	1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/09629

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 13-17
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 13-17 are directed to a method for treatment of the human body by therapy (Rule 39 PCT), the search has been carried out based on the alleged effects of the composition mentioned in the claims.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/09629

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9505452	23-02-95	AU-A- 7568094	14-03-95
		CA-A- 2169292	23-02-95
		FI-A- 960611	09-04-96
		NO-A- 960547	12-04-96

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